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A FUNCTIONAL ANALYSIS OF THE 3' REGULATORY REGION OF THE IMMUNOGLOBULIN HEAVY CHAIN GENE

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A FUNCTIONAL ANALYSIS OF THE 3' REGULATORY REGION OF THE IMMUNOGLOBULIN HEAVY CHAIN GENE

A dissertation submitted in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

By

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I HEREBY RECOMMEND THAT THE DISSERATION PREPARED UNDER MY SUPERVISION BY Andrew D. Snyder ENTITLED A Functional Analysis of the 3' Regulatory Region of the Immunoglobulin Heavy Chain Gene BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Snyder, Andrew D. Ph.D., Biomedical Sciences Program, Wright State University 2016
A Functional Analysis of the 3' Regulatory Region of the Immunoglobulin Heavy Chain Gene

The immunoglobulin heavy chain (*IGH*) locus is partially responsible for immunoglobulin (Ig) production in B cells. The human *IGH* locus contains two 3' regulatory regions (*3'IghRR*) that each contain three enhancers, which are thought to help drive overall transcription of the locus and also influence class switching to alternative Ig isotypes. The hs1.2 enhancer within the *3'IghRR* is polymorphic in humans, containing a 53 bp invariant sequence (IS) that can be repeated up to four times. In vitro, the human hs1.2 enhancer is a sensitive target of exogenous chemicals, particularly 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin), a potent inhibitor of Ig expression in animal models. The IS polymorphisms have also been associated with many immunological disorders in human patients. Therefore, understanding the role of the hs1.2 polymorphisms could be invaluable to human health. To investigate the function of the hs1.2 polymorphism, a mutational analysis was performed in a luciferase assay system to assess the contribution of each hs1.2 transcription factor binding site to overall hs1.2 activity. From this analysis, it is clear that each transcription factor binding site individually contributes to the changing activity of the hs1.2 enhancer with B-cell

stimulation. Surprisingly, results also indicate that TCDD likely acts on the hs1.2 enhancer through NF1 binding sites rather than dioxin response elements. However, further studies using luciferase reporters containing additional *3'IghRR* elements call into question the value of luciferase assays in assessing *3'IghRR* function. In order to study the endogenous *3'IghRR* directly, the CRISPR/Cas9 gene editing system was used to modify the hs1.2 enhancer in the human *3'IghRR*. Using a single CRISPR/Cas9 plasmid targeting the polymorphic hs1.2 invariant sequence (IS) repeats in a human B-cell line has successfully resulted in clonal populations containing a reduced number of IS repeats. Experiments with these cells have revealed that changing the combination of hs1.2 polymorphisms decreases the expression of some Ig isotypes while increasing others. Significantly, the α_1 hs1.2 enhancer has a surprisingly large effect on expression of ε transcripts, suggesting this enhancer should be investigated as a target of small molecule therapy in allergy sufferers.

TABLE OF CONTENTS

LIST OF FIGURES

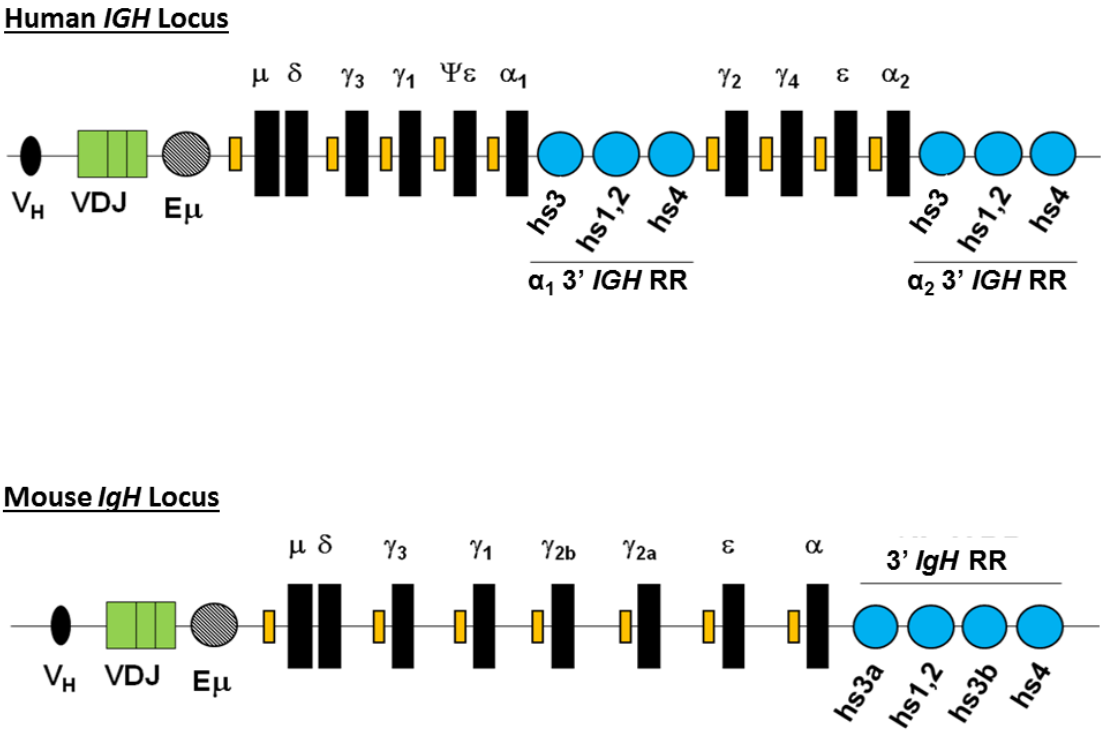
LIST OF TABLES

I. Literature Review

The production of antibodies, also called immunoglobulins (Ig), is a complex and highly regulated process that starts during B-cell maturation. The stages of B-cell maturation are characterized by the DNA recombination events that must occur in order to produce functional Ig. Functional Ig is formed by linking two light chain and heavy chain proteins that come to together to form a "Y" shaped molecule. The heavy chain of Ig (IGH) contains a variable region at one end, which forms one half of the antigen binding site, and a constant region at the other that determines how the molecule will function in different immunological contexts. The heavy chain of Ig is expressed by the *IGH* gene, which has a complex structure with the variable heavy chain (V_H) promoter at the 5' end followed by the variable (V), diversity (D), and joining (J) regions which encode the variable end of the IGH molecule. After the VDJ regions there is an intronic enhancer, Eμ, followed by the constant regions (i.e., C_γ, C_ε, etc.) that encode the different isotypes, or classes, of Ig and then the 3' IGH regulatory region (*3'IghRR*) which is the most 3' end of the gene (Fig. 1).

Before a B cell fully differentiates to express antibodies, it gets activated in either a T cell dependent or independent response. Once activated, either by T cells or an antigen, the B cell differentiates into a plasma cell and begins secreting large amounts of Ig. In order for productive transcripts of IGH to be produced, the IGH gene must first undergo VDJ recombination. VDJ recombination is a process used to assemble the separated V, D, and J segments and ensures the diversity of antigen binding sites in Ig. This process begins with D-J rearrangement in the pre- B-cell stage followed by V-DJ

A



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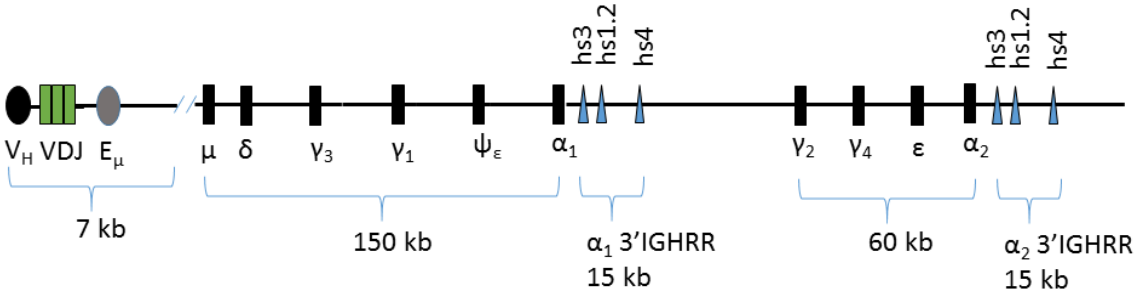

Figure 1. Human versus mouse *IGH* locus (A). The *IGH* locus is highly homologous between mice and humans, but still contains significant structural differences. The human locus contains two *3'IghRR*s whereas the mouse only contains one. The mouse locus also contains an additional enhancer. A second diagram of the human locus (B) reflects the overall size of the human *IGH* locus drawn roughly to scale. The broken line following the E_μ enhancer represents a change in scale between the first part of the diagram and the rest.

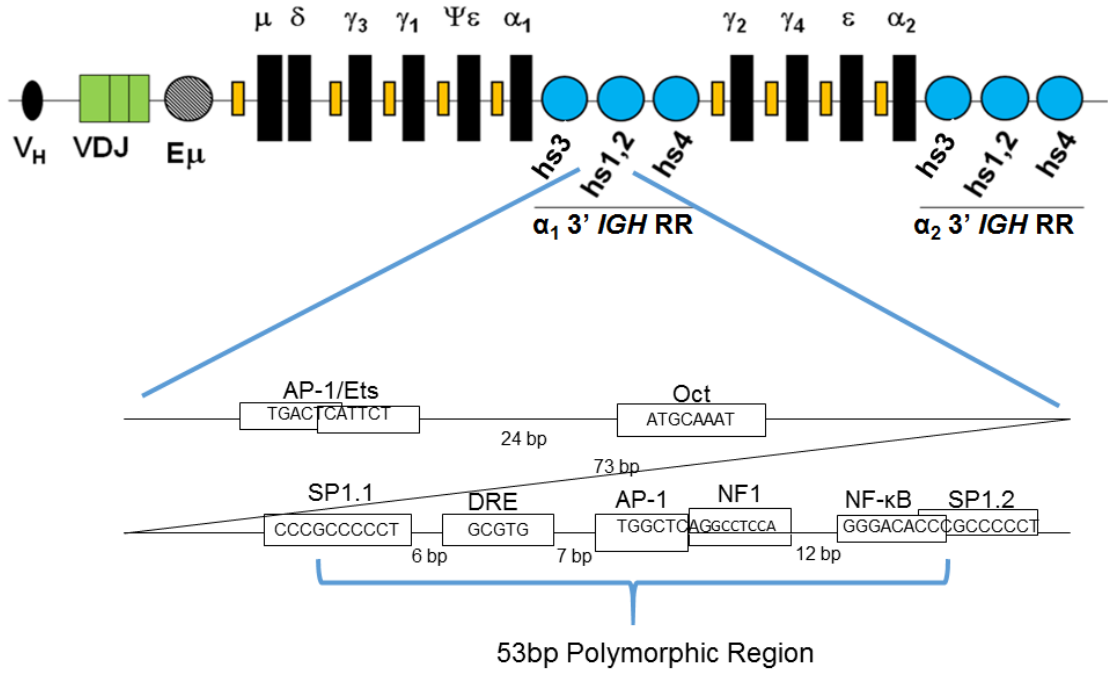

Figure 2. Human *IGH* locus with hs1.2 enhancer. The hs1.2 enhancer is densely packed with transcription factor binding sites. A 53 base pair invariant sequence (IS) is polymorphic and can be repeated up to four times.

rearrangement in the pro-B-cell stage. This process is mediated by Rag1/2 and involves making a double strand break in the DNA which necessitates activation of the DNA repair machinery (reviewed in (Soulas-Sprauel et al., 2007)). Any B-cells that fail to recombine successfully undergo apoptosis and are eliminated. During an active immune response, in activated (stimulated) B cells, VDJ recombination can be followed by somatic hypermutation of the variable region which further increases the affinity of the antibody for the activating antigen. In the later stages of the humoral immune response the Ig heavy chain locus can undergo class switch recombination. Whenever a B cell is going to produce a type of Ig other than IgM the cell needs to undergo class switch recombination in order to alter the effector function of the Ig without changing its antigen specificity. This step involves the repetitive switch regions upstream of each constant region and is initiated by activation-induced cytidine deaminase. Breaks are introduced into the DNA at the two switch regions, which then fuse together, and the intervening region is excised as a circular piece of DNA which then gets digested (Fig. 3) (Dudley, Chaudhuri, Bassing, & Alt, 2005; Soulas-Sprauel et al., 2007).

A major regulator of transcription of IGH as well as class switch recombination and somatic hypermutation is the *3'IghRR*, a roughly 20 kb region found downstream of the constant regions (Lieberson, Ong, Shi, & Eckhardt, 1995; Mills, Harindranath, Mitchell, & Max, 1997; Pinaud et al., 2001; Pinaud et al., 2011; Vincent-Fabert et al., 2010). The mouse *3'IghRR* is composed of four DNase I hypersensitive sites that exhibit enhancer activity, while in humans there are only three enhancers, which are duplicated. The human *3'IghRRs* are distinguished as the α_1 *3'IghRR*, which sits

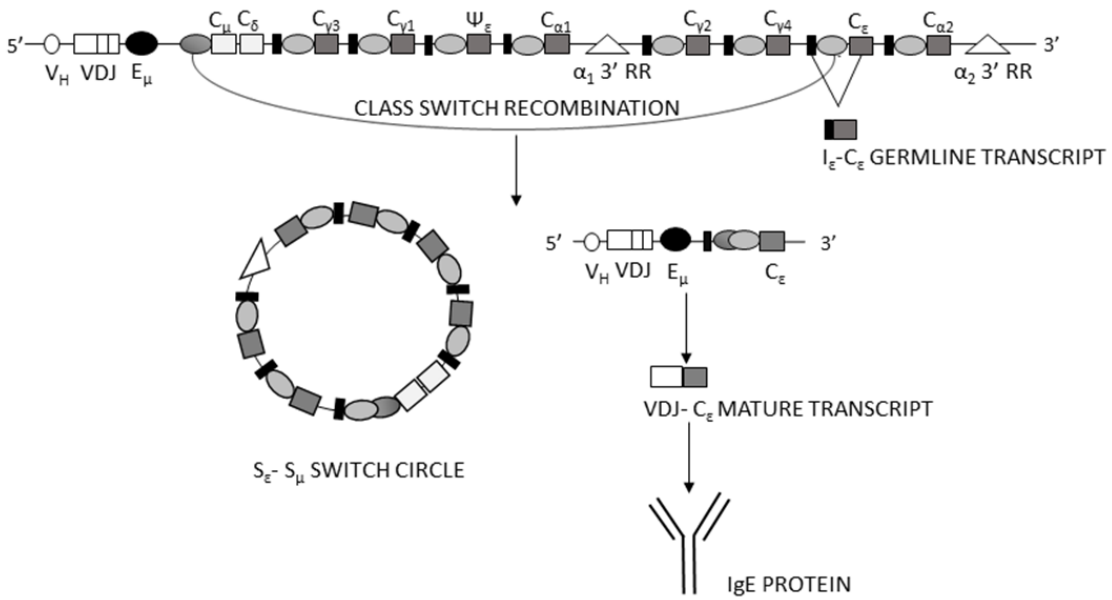

Figure 3. Class switch recombination. During class switch recombination the *3'IghRR* is thought to drive transcription of a sterile germline transcript which increases the availability of the switch regions (grey ovals). When the switch regions are joined together a double strand break occurs and the intervening sequence is excised as a switch circle. The new constant region (ε in this example) takes the place of the μ constant region and a functional transcript is created from the recombined locus. Figure reproduced from (Burra, 2015).

downstream of $C\alpha_1$, and the *α_2 3'IghRR*, which sites downstream of $C\alpha_2$ and marks the 3' end of the IGH gene (Mills et al., 1997; Pinaud, Aupetit, Chauveau, & Cogne, 1997). These regions are weak enhancers individually, but together exert strong control over transcription and class switch recombination of *IGH* (Chauveau, Pinaud, & Cogne, 1998). The enhancers are dispersed among long stretches of highly repetitive, somewhat palindromic sequences which may also be functional, although this idea has yet to be systematically tested (D'Addabbo, Scascitelli, Giambra, Rocchi, & Frezza, 2011). The *3'IghRR* enhancers each contain numerous transcription factor binding sites for widely studied transcription factors such as NFκB, Oct, and AP-1 (Pinaud et al., 2011). The enhancer that contains the most transcription factor binding sites is hs1.2, which contains a 53 bp invariant sequence that is polymorphic, meaning it can appear a different number of times in different people (Fig. 2) (Chauveau et al., 1998; Guglielmi, Truffinet, Magnoux, Cogne, & Denizot, 2004; Mills et al., 1997; Pinaud et al., 1997). This invariant sequence can be repeated up to four times (Fig. 2), and these repeats have been associated with some human disease states (Chauveau et al., 1998; Cianci et al., 2008; Frezza et al., 2009; Frezza et al., 2007; Mills et al., 1997; Pinaud et al., 1997; Tolusso et al., 2009). The polymorphism in the human hs1.2 enhancer does not exist in mice, and there are other species differences between mice and humans. For example, the mouse *3'IghRR* contains Pax5 binding sites, while the human *3'IghRR* does not (Pinaud et al., 2011). The significant structural differences between the mouse and human locus, coupled with the presence of different assortments of transcription factor

binding sites between the two species, could indicate that the *3'IghRR* functions differently in the two species.

The mouse *3'IghRR* is a sensitive target of exogenous chemicals and treatment with these chemicals can lead to altered Ig expression (Fernando, Ochs, Liu, Chambers-Turner, & Sulentic, 2012; Sulentic, Kang, Na, & Kaminski, 2004; Wourms & Sulentic, 2015). TCDD, a common environmental pollutant, is a potent inhibitor of Igh in animal models (reviewed in (Hanieh, 2014; Quintana, 2013)). Humans exposed to TCDD exhibit a variety of toxicological effects including chloracne, liver dysfunction, carcinogenesis, and cardiovascular diseases (Marinkovic, Pasalic, Ferencak, Grskovic, & Stavljenic Rukavina, 2010; McGregor, Partensky, Wilbourn, & Rice, 1998; Pesatori et al., 2003; Yu, Guo, Hsu, & Rogan, 1997; Zober, Ott, & Messerer, 1994). The effect of TCDD on the human immune system, particularly in B cells, is still largely unknown. However, in mouse models TCDD is a potent immunosuppressant (Denison & Nagy, 2003; Denison, Pandini, Nagy, Baldwin, & Bonati, 2002). Many studies have shown that TCDD binds to the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor which can bind to a variety of synthetic and natural ligands (Y. Fujii-Kuriyama & Mimura, 2005; Noakes, 2015; Schecter, Birnbaum, Ryan, & Constable, 2006). TCDD is the strongest AhR ligand currently known (C. Vogel et al., 1997). Once bound to TCDD, the AhR sheds its associated chaperone proteins and translocates to the nucleus where it binds to the AhR nuclear translocator (ARNT) (Yoshiaki Fujii-Kuriyama & Kawajiri, 2010; Y. Fujii-Kuriyama & Mimura, 2005). The TCDD-AhR-ARNT complex then binds to dioxin response elements (DRE) found in sensitive genes (Fig.2) (Yoshiaki Fujii-Kuriyama & Kawajiri, 2010; Y. Fujii-

Kuriyama & Mimura, 2005). This molecular pathway has been most extensively studied in relation to the DRE binding sites in CYP1A1, but recent work has also shown AhR binding to the DRE within the hs1.2 enhancer of the mouse *3'IghRR* following TCDD treatment (Y. Fujii-Kuriyama & Mimura, 2005; Wourms & Sulentic, 2015). Rodents display a variety of innate and acquired immune-related disturbances following acute or chronic exposure to low levels of TCDD (Holsapple, Morris, Wood, & Snyder, 1991; Vos, De Heer, & Van Loveren, 1997). TCDD affects B-cell maturation, activation, differentiation and to a lesser extent proliferation (Sulentic & Kaminski, 2011). Importantly, this work was done in mouse B cells and it is currently unknown if the same mechanism is at work in human B cells. TCDD has also been shown to inhibit differentiation of B cells by altering DNA methylation patterns (McClure, North, Kaminski, & Goodman, 2011).

Past research shows that mouse *3'IghRR* transcriptional activity in LPS-stimulated CH12.LX mouse B cells is inhibited by TCDD (Sulentic et al., 2004). This effect parallels TCDD-induced inhibition of μ heavy chain gene expression and IgM production (Sulentic, Holsapple, & Kaminski, 2000). The hs4 and hs1.2 enhancers each contain a DRE-like site that demonstrates TCDD-inducible binding of AhR/ARNT by EMSA-Western and ChIP analysis (Salisbury & Sulentic, 2015; Sulentic et al., 2000), which supports at least a partial role of DRE-dependent regulation; however, the AhR is known to interact directly and indirectly with a number of different transcription factors such as AP-1, NF-κB, and SP-1 (Kobayashi, Sogawa, & Fujii-Kuriyama, 1996; Suh et al., 2002; Tian, 2009; Tian, Ke, Denison, Rabson, & Gallo, 1999). Even though a link between the AhR and Oct

remains unclear, a high frequency of Oct sites was found in AhR-responsive genes using a genetic algorithm, thus suggesting a potential role for Oct in mediating a response to TCDD (Kel et al., 2004). Interestingly, Oct and NF-κB act as repressors of mouse hs1.2 activity in mature, non-activated B cells but become activators of mouse hs1.2 activity in plasma cells suggesting a cooperative interaction between Oct and NF-κB and a potential modulation by TCDD or the AhR (Michaelson et al., 1996; Salisbury & Sulentic, 2015; Wourms & Sulentic, 2015).

A few studies have been published regarding epigenetics of the *3'IghRR*. Studies have shown that histones H3 and H4 in hs4 are demethylated during B-cell maturation and hs3b and hs1.2 are demethylated after B-cell activation (Garrett et al., 2005). Further chromatin immunoprecipitation experiments have shown a stepwise activation of the *3'IghRR* with histone H3 in hs4 becoming acetylated in the pro- and pre- B-cell stages while hs3, hs1.2, and hs3b become acetylated in mature B-cells (Garrett et al., 2005). However, these observations are difficult to apply to the general function of the *3'IghRR* enhancers, because the effect of deletions of these enhancers in various model systems do not seem to correlate to specific stages of B-cell maturation. In fact, deletions of each enhancer individually in mouse models showed no significant alteration in transcription of any Igh isotype except IgM (Bebin et al., 2010; Cogne et al., 1994; Manis et al., 1998; Vincent-Fabert et al., 2009). In contrast, deletion of the *3'IghRR* enhancers in pairs resulted in a marked decrease in transcription and class switch recombination (Dunnick et al., 2009; Dunnick, Shi, Graves, & Collins, 2005). Deletion of the entire mouse *3'IghRR* resulted in a broad reduction of Igh expression,

class switch recombination, and alterations to somatic hypermutation (Vincent-Fabert et al., 2010). Chromosome conformation capture experiments have shown that the *3'IghRR* enhancers physically interact with the V_H promoter and the Eμ intronic enhancer, although Eμ is dispensable for the interaction of the *3'IghRR* with the promoter (Ju, Chatterjee, & Birshtein, 2011). The looping of the *3'IghRR* also leads it to interact with the J_H region, and this looping occurred independently of expression of several transcription factors which bind to the *3'IghRR* (Ju et al., 2011). To reiterate, much of this research was conducted using mouse models, but due to the significant differences between the mouse and human *3'IghRR*, it is difficult to know how much of it translates between the two species. Therefore, more research in human model systems is clearly necessary, but new tools are needed to conduct these studies since technical limitations and lack of appropriate cellular models have hampered study progress. Recent advances in molecular biology have made it possible to create genetic modifications that were impossible just a few years ago. Specifically, the development of the CRISPR genetic editing system makes it possible to create highly specific, targeted genetic edits in virtually any living cell (He et al., 2015; Li et al., 2015; Mali et al., 2013; Zhang et al., 2015; Zheng et al., 2014). Taking advantage of the development of this new gene editing technique makes it possible to study the function of the endogenous human *3'IGHRR* in a way that has never been done before so that significant gaps in knowledge about the human *3'IGHRR* can begin to be addressed.

In spite of the decades of research conducted on the Ig heavy chain locus there are still significant unknowns associated with the function of this gene. The functional

significance of the many structural differences between the mouse and human *3'IghRR* is still unknown. For example, the duplication of the *3'IghRR* in the human locus is thought to be functionally significant, but no studies have investigated the specific roles of the α_1 versus α_2 *3'IghRR* in antibody expression. Logically, since the first *3'IghRR* is lost in the switch circle during class switch recombination to downstream constant regions, it seems likely that the second *3'IghRR* should be able to compensate for loss of the first. However, no studies have been conducted to support this theory. Another major structural difference between the mouse and human *3'IGHRR* is found in the hs1.2 enhancer. The human hs1.2 enhancer contains a 53 bp invariant sequence (IS) which is polymorphic and can be repeated up to four times. This polymorphism does not exist in the mouse hs1.2 enhancer, so all studies involving the mouse *Igh* locus have missed any functional significance of the human polymorphism. The human hs1.2 enhancer also contains a completely different complement of transcription factor binding sites, which raises the possibility that the mouse and human hs1.2 enhancers could function in different ways. The goal of this research is to confirm the findings of the animal studies by demonstrating that the human *3'IGHRR* is a powerful regulator of IGH expression while also attempting to determine the functional significance of the hs1.2 polymorphism.

Significance

Polymorphisms within the α_1 *3'IghRR* hs1.2 enhancer have been associated with a host of immune disorders such as rheumatoid arthritis, dermatitis herpetiformis,

systemic sclerosis, and celiac disease (Cianci et al., 2008; Frezza et al., 2009; Frezza et al., 2007; Tolusso et al., 2009). Also, chromosomal translocations between the *3'IghRR* and c-myc have been known to lead to cancer, especially Burkitt's lymphoma (J. Wang & Boxer, 2005). Since genetic variations in the *3'IghRR* are associated with so many human diseases, a better understanding of its function and regulation by exogenous chemicals could be immensely beneficial to human health. Furthermore, this research could lead to better or more targeted treatments of immunological conditions associated with *3'IghRR* polymorphisms or translocations because it is thought to be such a potent regulator of overall IGH expression based on animal studies. For example, allergy treatments in particular might be improved by a better understanding of the *3'IghRR*. Establishing how the human *3'IghRR* works, in particular how it responds to exogenous chemicals such as TCDD, could benefit many people suffering from an immunological medical condition and even some cancers.

II. Specific Aim 1

Specific Aim 1 Rationale

The *3'IghRR* has been extensively studied in mouse model systems and its functions as a key regulator of transcription, class switch recombination, and somatic hypermutation are well established (Pinaud et al., 2011). However, relatively little work has been done to evaluate the human *3'IghRR*. There are significant species differences between the human and mouse *IGH* genes, the most obvious of which is the duplication of the human *3'IghRR*. Other differences between mouse and human *3'IghRR* include significant differences in the transcription factor binding sites within the individual enhancers that make up the *3'IghRR* and the presence of additional DNase I hypersensitive sites downstream of the enhancers in the mouse *3'IghRR* (Mills et al., 1997; Pinaud et al., 1997; Sepulveda, Garrett, Price-Whelan, & Birshtein, 2005). Although most of the literature appears to assume the mouse and human *3'IghRR*s function in the same way, the significant structural differences between the two species suggest there could also be functional differences. These facts make it difficult to apply murine research to the human *3'IghRR*, which reinforces the necessity of more clearly establishing how the human *3'IghRR* operates.

The human hs1.2 enhancer within the *3'IghRR* has a completely different complement of transcription factor binding sites than the mouse hs1.2 enhancer. This pronounced difference in transcription factors could suggest a species difference in how the hs1.2 enhancer operates, and to begin to understand this potential difference it is necessary to evaluate the function of the hs1.2 enhancer's many transcription factor binding sites.

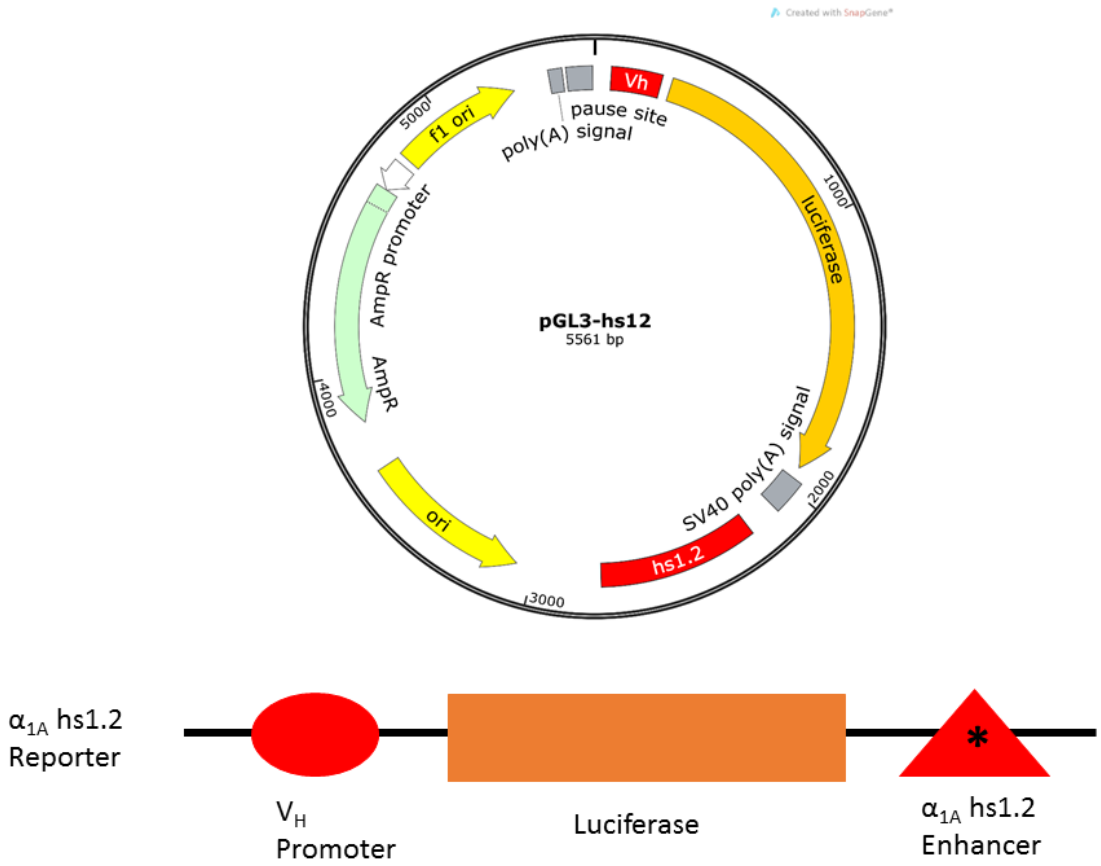

Figure 4. The hs1.2 luciferase reporter plasmid. The hs1.2 luciferase reporter plasmid contains an hs1.2 enhancer with a single polymorphic invariant sequence (IS) and the variable heavy chain promoter (V_H). Transcription factor binding sites in the hs1.2 enhancer were mutated through site directed mutagenesis and used in transfection studies.

Experiments for specific aim 1 will utilize the CH12.LX mouse B cell line. The CH12.LX cell line was derived from the murine CH12 B-cell lymphoma arising in a B10.H-2aH-4bp/Wts (2a4b) mouse. The CH12.LX cell line was characterized by Bishop and Haughton (1986) and has been used extensively in immunological and toxicological research (Haughton, Arnold, Bishop, & Mercolino, 1986). The CH12.LX cells have high AhR expression and a functional AhR signaling pathway. These cells also demonstrate a sensitive inhibition of LPS-induced Ig expression by TCDD as seen in vivo and in primary B cells (Sulentic, Holsapple, & Kaminski, 1998; Sulentic et al., 2000). To determine the relative contribution of each transcription factor binding site to the activity of the human α_1 hs1.2 enhancer, site directed mutagenesis of a luciferase reporter containing the human *3'IghRR* α_1 hs1.2 enhancer was conducted and the resultant mutants were analyzed under different treatment conditions in the well-characterized CH12.LX mouse B-cell line to test the hypothesis that the DRE within the hs1.2 enhancer mediates the enhancer's response to TCDD and that transcription factor binding sites outside the IS polymorphism have a stronger effect on overall and stimulation-induced transcriptional activity.

Specific Aim 1 Results

Mutations of the binding sites outside of the IS have opposite effects

The AP1.Ets binding site is the most 5' transcription factor binding site in the hs1.2 enhancer and is not one of the sites repeated in the hs1.2 polymorphism. There is

a single nucleotide difference between the mouse and human AP1.Ets site, which causes the human AP-1 site to match the consensus sequence (Mills et al., 1997). In the murine hs1.2 enhancer the AP1.Ets site confers responsiveness to B-cell receptor cross-linking and has a functional role in *3'IghRR* activity (Grant, Thompson, & Pettersson, 1995). Following IgM receptor activation of primary B lymphocytes or BAL-17 cells, a mouse B cell lymphoma cells line, enhancer activation was concurrent with recruitment and binding of nuclear factor of activated B cells (NFAB) to the AP1.Ets site (Grant et al., 1995). Correspondingly, mutation of the AP1.Ets site had the most dramatic impact on overall transcription as compared to the other transcription factor binding site mutations. The mutation of the AP1.Ets site nearly eliminated overall transcriptional activity by the hs1.2 enhancer. This was true in both LPS-stimulated and unstimulated cells. Interestingly, TCDD-induced fold-activation of the enhancer was unaffected by this mutation. This result suggests the AP1.Ets site strongly regulates hs1.2 enhancer activity but plays no role in TCDD-induced activation of this enhancer (data not shown, refer to (Ochs, 2012)).

Like the AP1.Ets site the transcription factor octamer (Oct) is located 5' of the invariant sequence (IS). Oct contributes to mouse hs1.2 enhancer activity and is conserved between mouse and human (Mills et al., 1997). In B cells, Oct in collaboration with G-rich, κB-like motifs and Pax5 repress transcription of the murine hs1.2 enhancer (Singh & Birshtein, 1996). Our results suggest Oct may play a similar role in the human hs1.2 enhancer because mutation of the Oct binding site increased overall activity of the hs1.2 enhancer. Mutation of the Oct binding site also significantly increased TCDD-

induced activation of the hs1.2 enhancer in terms of fold change, suggesting that the Oct site suppresses the TCDD response. However, this effect was only observed in unstimulated cells, which indicates some aspect of TLR-4 signaling can override the effect of Oct on TCDD-induced activation (data not shown, refer to (Ochs, 2012)).

Mutation of any binding site within the IS increases overall transcriptional activity by the hs1.2 enhancer

There are two SP-1 binding sites present in the human hs1.2 enhancer which are found straddling either end of the approximately 53 bp polymorphic IS. These SP-1 sites are not conserved between the mouse and human locus, and are not repeated in the human polymorphisms. There is no information available about the role SP-1 plays in IGH regulation, but SP-1 is often associated with other transcription factors, especially those which have binding sites in the hs1.2 enhancer (Kobayashi et al., 1996; Nehls, Rippe, Veloz, & Brenner, 1991; Rafty, Santiago, & Khachigian, 2002; Safe & Abdelrahim, 2005; Tapias, Ciudad, & Noe, 2008; F. Wang, Wang, & Safe, 1999). In particular, other groups have shown interactions between SP-1 and NF1 and also between SP-1 and AhR (Kobayashi et al., 1996; Nehls et al., 1991; Rafty et al., 2002; F. Wang et al., 1999). Mutation of either SP-1 site significantly increased overall transcriptional activity by the hs1.2 enhancer but had no significant effect on the TCDD-induced activation of the enhancer in terms of fold change over the vehicle control (Fig. 5 and 6).

The IS within the hs1.2 enhancer contains four transcription factor binding sites; AP-1, NF1, NF-κB, and a DRE core binding motiff. Most of these sites are repeated up to

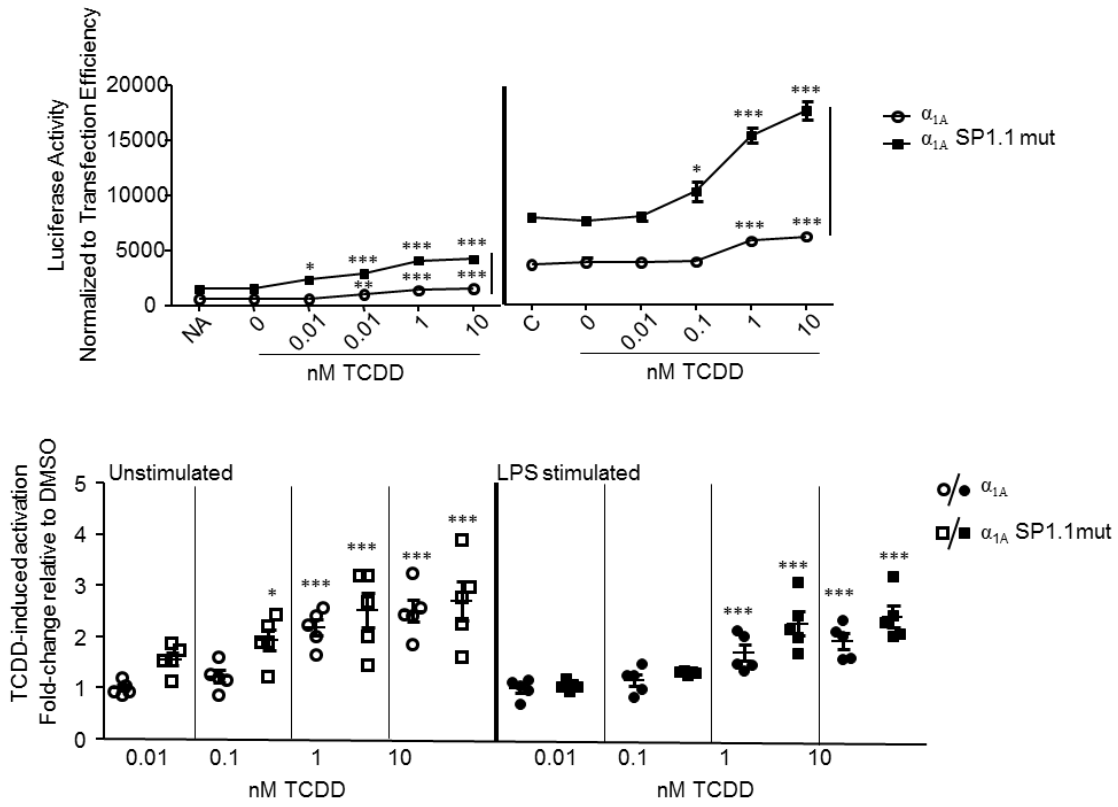

Figure 5. Mutational analysis of the SP1 transcription factor binding sites within the hs1,2 enhancer. CH12.LX cells were transiently transfected with the hs1.2 reporter plasmid with the 5' SP1 site (SP1.1) mutated. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.001-10.0 nM) in the absence or presence of LPS (0.1 μg/ml) stimulation for 24 hours. Luciferase enzyme activity (mean ± SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency (top graph.) Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of at least three independent experiments (bottom graph.) Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. One, two, or three asterisks (*) denote significance compared to the corresponding vehicle control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA followed by a Bonferroni post-test. "†", "††", "†††", denote significant differences between reporter plasmids for each treatment at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. A vertical line represents a significant difference between reporter plasmids for all treatment groups of at least $p<0.01$.

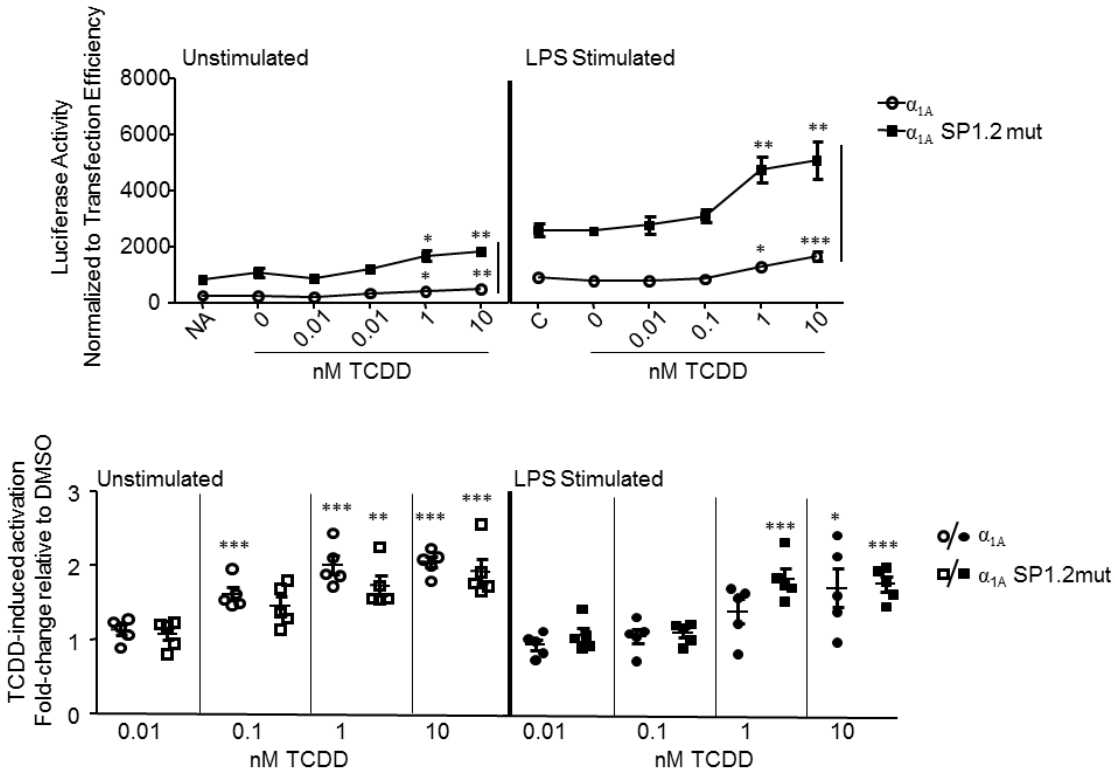

Figure 6. Mutational analysis of the SP1 transcription factor binding sites within the hs1,2 enhancer. CH12.LX cells were transiently transfected with the hs1.2 reporter plasmid with the 3' SP1 site (SP1.2) mutated. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.001-10.0 nM) in the absence or presence of LPS (0.1 μg/ml) stimulation for 24 hours. Luciferase enzyme activity (mean ± SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency (top graph.) Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of at least three independent experiments (bottom graph.) Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. One, two, or three asterisks (*) denote significance compared to the corresponding vehicle control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA followed by a Bonferroni post-test. "†", "††", "†††", denote significant differences between reporter plasmids for each treatment at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. A vertical line represents a significant difference between reporter plasmids for all treatment groups of at least $p<0.01$.

four times in the IS sequence polymorphisms. Since previous studies have demonstrated an AhR-dependant activation of the human hs1.2 enhancer by TCDD (Fernando et al., 2012), the identification of a DRE core motif in the repeated IS sequence seemed a plausible route for this effect. Additionally, it has been shown that AP-1 and NF-κB are affected by the AhR or TCDD through cross-talk interactions or altered binding and expression, so mutation of these sites was also expected to affect TCDD-induced enhancer activation (Suh et al., 2002; Tian, Rabson, & Gallo, 2002). Surprisingly, none of these sites when mutated were found to significantly affect TCDD-induced activation, not even the DRE (Fig. 7 and (Ochs, 2012)). However, mutation of these binding sites did significantly, but modestly, increase overall transcriptional activity of the hs1.2 enhancer, which stands in stark contrast to our previous study that found deletion of the IS lead to a large decrease in overall hs1.2 activity (Fernando et al., 2012). These results underscore the complexity of the hs1.2 transcriptional regulation and suggest that TCDD modulates hs1.2 enhancer activity through a non-canonical pathway.

Mutation of the NF1 binding site reduces TCDD-induced activity but has little effect on overall transcriptional activity

The nuclear factor 1 binding site is located in the middle of the IS and is repeated in the hs1.2 polymorphisms. Little is known about the role of NF1 in IGH regulation, but there is evidence that NF1 functions cooperatively with other hs1.2 transcription factors, in particular SP-1 (Nehls et al., 1991). However, some of this work has been contradictory (Rafty et al., 2002). To our knowledge, no one has shown a direct

relationship between NF1 and AhR. In the current study, mutation of the NF1 site had little effect on overall transcriptional activity of the hs1.2 enhancer as compared to mutation of any of the other transcription factor binding sites. However, an unexpected result of the NF1 mutation was a significant reduction in TCDD-induced activation of the hs1.2 enhancer in terms of fold change over the vehicle control (Fig. 8). Interestingly, this reduction only occurred in LPS-stimulated B cells and not in unstimulated cells. This result further supports our conclusion that TCDD affects hs1.2 enhancer activity through a non-canonical AhR-mediated pathway.

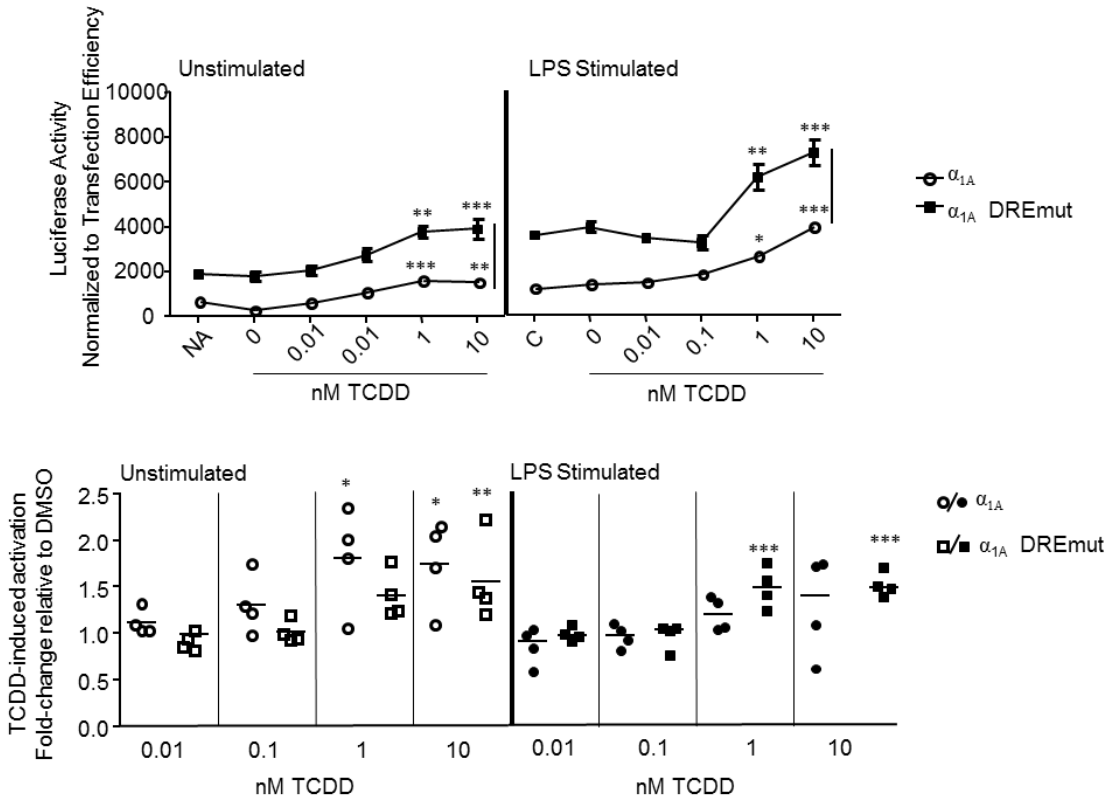

Figure 7. Mutational analysis of the DRE transcription factor binding site within the hs1,2 enhancer. CH12.LX cells were transiently transfected with the hs1.2 reporter plasmid with the DRE site mutated. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.001-10.0 nM) in the absence or presence of LPS (0.1 μg/ml) stimulation for 24 hours. Luciferase enzyme activity (mean ± SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency (top graph.) Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of at least three independent experiments (bottom graph.) Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. One, two, or three asterisks (*) denote significance compared to the corresponding vehicle control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA followed by a Bonferroni post-test. "†", "††", "†††", denote significant differences between reporter plasmids for each treatment at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. A vertical line represents a significant difference between reporter plasmids for all treatment groups of at least $p<0.01$.

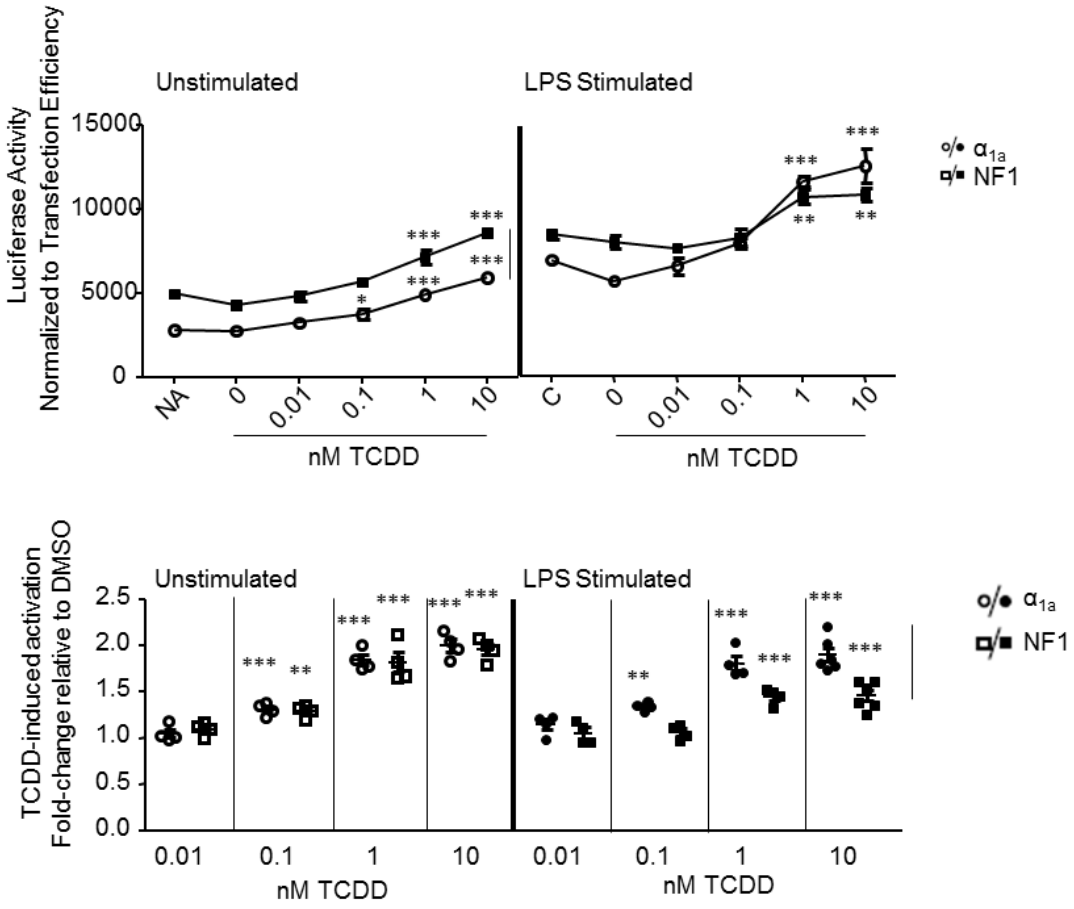

Figure 8. Mutational analysis of the NF1 transcription factor binding site within the hs1,2 enhancer. CH12.LX cells were transiently transfected with the hs1.2 reporter plasmid with the NF1 site mutated. Transfected cells were either cultured in the absence of any additional treatment (naïve, NA) or treated for 24 h with 0.01% DMSO vehicle (0 nM TCDD) or TCDD (0.001-10.0 nM) in the absence or presence of LPS (0.1 μg/ml) stimulation for 24 hours. Luciferase enzyme activity (mean ± SEM, n=3) is represented on the y-axis as relative light units normalized to transfection efficiency (top graph.) Fold change is represented on the y-axis relative to the respective DMSO vehicle control and was generated from averaging the means of at least three independent experiments (bottom graph.) Comparisons between treatment groups of the same plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. One, two, or three asterisks (*) denote significance compared to the corresponding vehicle control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively. Comparisons between reporter plasmids were analyzed using a 2-way ANOVA followed by a Bonferroni post-test. "†", "††", "†††", denote significant differences between reporter plasmids for each treatment at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. A vertical line represents a significant difference between reporter plasmids for all treatment groups of at least $p<0.01$.

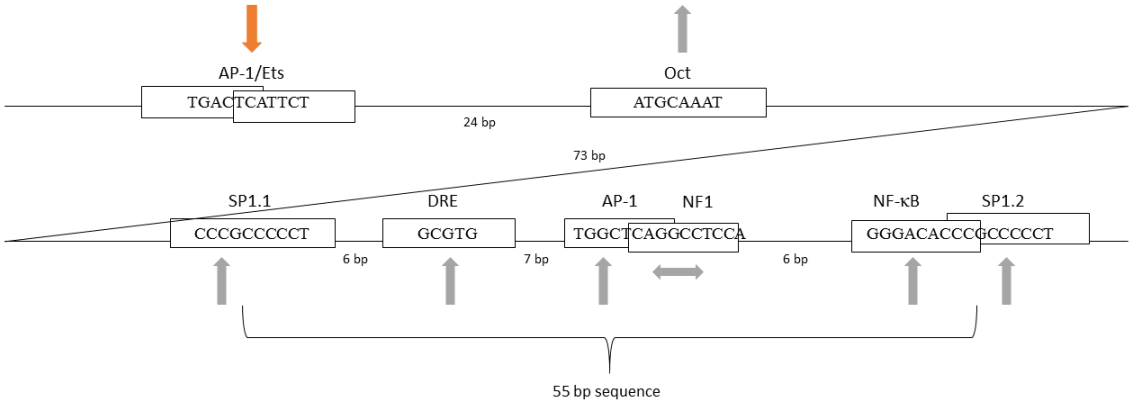

Figure 9. Summary of the impact of specific mutations on human hs1.2 reporter activity. Up or down arrows means more or less overall transcriptional activity compared to the non-mutated plasmid. Treatment with TCDD or LPS caused an increase in reporter activity regardless of which binding site was mutated. Only the mutated NF1 binding site lead to a significant decrease in the TCDD response, and only when the cells were stimulated by LPS.

III. Specific Aim 2

Specific Aim 2 Rationale

Most current knowledge of the *3'IghRR* has been derived from studies in mouse models due to the difficulty of studying this region in human cells. The human *3'IghRR* duplication makes it difficult to discern which *3'IghRR* is responsible for IGH expression. Also, the vast amount of sequence identity between the α_1 and α_2 *3'IghRR* has made it nearly impossible to make specific deletions to either region using traditional genetic editing methods that require long arms of homology. For these reasons most of the human *3'IghRR* studies have been based on reporter plasmids (Pinaud et al., 2011). Unfortunately, these reporter plasmid studies have had significant deficiencies. So far all of these studies have either used separate plasmids for each enhancer, or have all the enhancers strung together without the intervening sequences (Fernando et al., 2012; Mills et al., 1997; Sulentic et al., 2004). Neither one of these situations reflects how the enhancers are situated in the cell, where the enhancers are thought to cooperatively interact and are separated by many kilobases of intervening sequence. Furthermore, studies involving the mouse and human hs1.2 enhancer have shown significantly different results that appear to be species dependent (Fernando et al., 2012). The mouse hs1.2 enhancer increases activity in response to B-cell stimulation and decreases activity in response to TCDD, which is consistent with theories of *3'IGHRR* function since B-cell stimulation is expected to increase *3'IGHRR*-driven transcription and TCDD is a known immunosuppressant in rodents. However, the human hs1.2 enhancer actually decreases activity in response to B-cell stimulation and increases activity in response to TCDD, which is exactly the opposite of the expected outcome (Fig. 10 and (Fernando et

al., 2012). The human hs1.2 enhancer also reacts differently to B-cell stimulation depending on if it's being studied in mouse or human B-cells (Fig. 10). One possible explanation for these unexpected results could be that the hs1.2 enhancer was taken out of its natural context i.e. lacking the other two enhancers. It is also possible that the intervening sequence between the enhancers could be functional since it contains multiple potential transcription factor binding sites as well as unique repetitive nucleotide sequences that some scientists believe might contribute to essential secondary structures that contribute to *3'IghRR* function (Giambra et al., 2005). Indeed, recent work using knockout mice that lack these intervening sequences have shown they have a significant effect on Ig expression (Garot et al., 2016).

Experiments in support of this specific aim utilized the human B-cell line CL-01 and the mouse B cell line CH12.LX. The CL-01 cell line expresses surface IgM and IgD, secretes monoclonal antibodies, and can be induced to undergo class switch recombination to multiple isotypes by TLR 9 stimulation or CD40L plus IL-4 (Cerutti et al., 1998; Zan et al., 1999). This is a Burkitt's lymphoma cell line, bearing the chromosomal translocation of one *3'IghRR* allele to the c-myc gene. It is currently unknown which allele has translocated to c-myc, which could complicate data interpretation. Regardless, the CL-01 cell line is better suited to this project than other human B-cell lines such as IM-9 due to the CL-01's ability to respond to stimulation and undergo class switch recombination, which are essential for this study. The unexpected results from human hs1.2 enhancer reporter studies combined with the lack of studies with the enhancers in their natural context coupled with the recent work on knockout

mice provides an excellent rationale for creating a new reporter plasmid with the human enhancers that includes more of the human *3'IghRR* sequence to test the hypothesis that a human *3'IghRR* reporter containing all the enhancers along with the intervening sequences between the enhancers will exhibit increased activity in response to B-cell stimulation and decreased activity in response to TCDD in human cells.

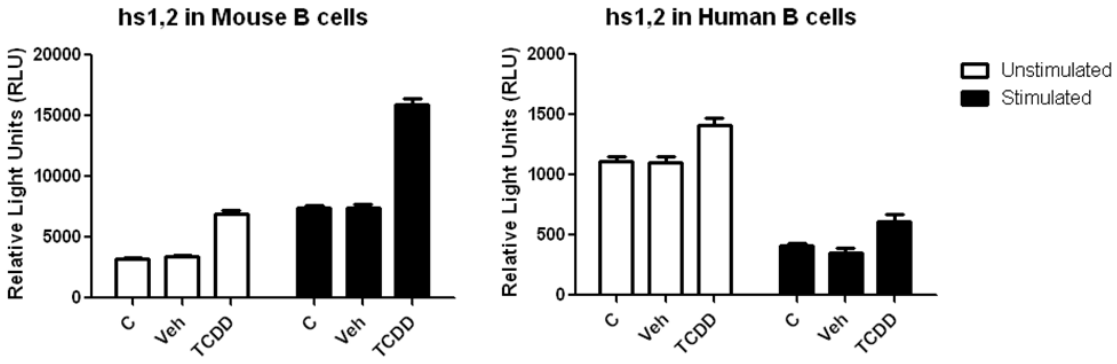

Figure 10. The hs1.2 reporter plasmid in mouse versus human cells. When the hs1.2 reporter plasmid is transfected into mouse CH12.LX cells which are stimulated by LPS, activity increases relative to unstimulated cells. Treatment with TCDD increases activity in both stimulated and unstimulated mouse cells. When the same reporter plasmid is transfected into human CL-01 cells which are stimulated with CD40L and IL-4, activity decreases relative to unstimulated cells. TCDD treatment increases activity under both conditions.

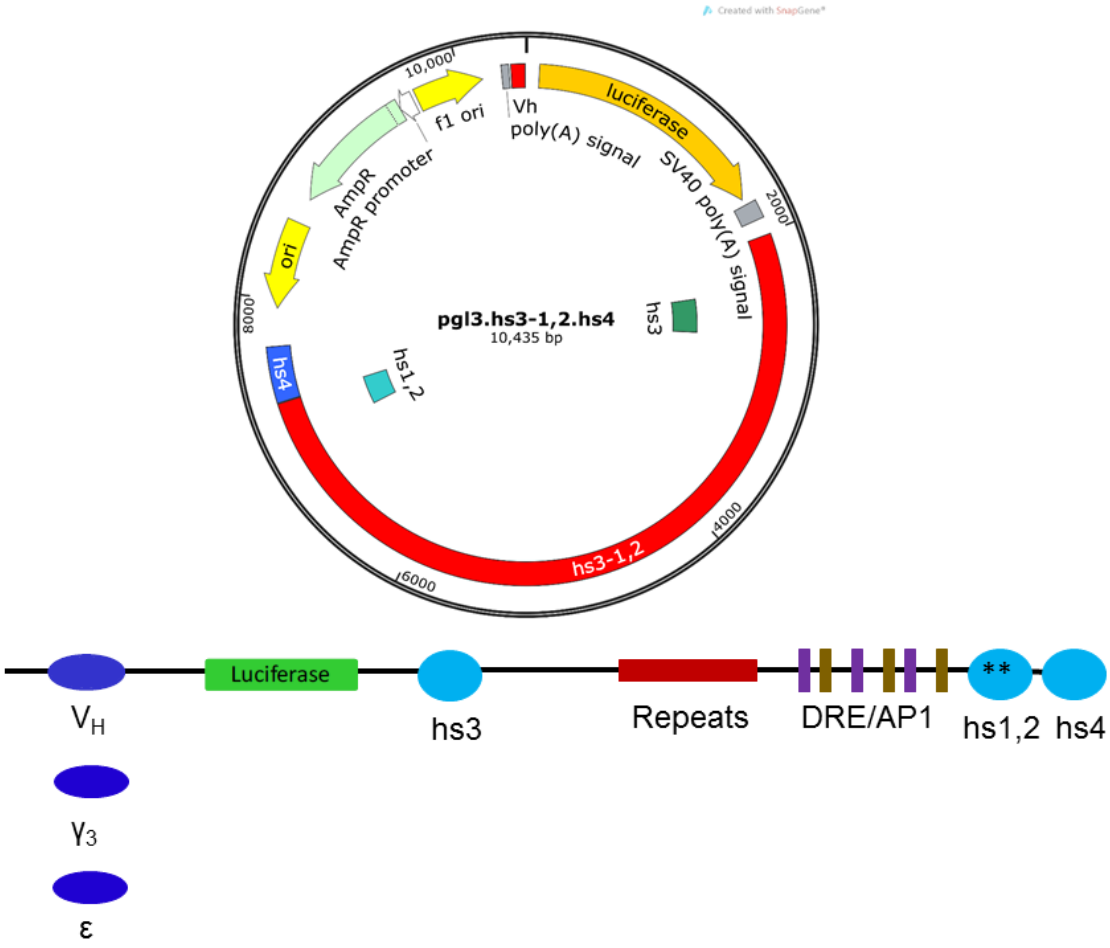

Figure 11. The pGL3.hs3-1.2.4 luciferase reporter plasmid. This new human *3'IghRR* reporter plasmid contains more of the *3'IghRR*, including the entire intervening sequence between hs3 and hs1.2. The hs1.2 enhancer contains two repeats of the polymorphic region. The hs4 enhancer follows directly after the hs1.2 enhancer. The intervening sequence between hs3 and hs1.2 contains long nucleotide repeats (microsatellite repeats) and additional DRE and AP-1 transcription factor binding sites. Variations of this plasmid were created which contained either the γ_3 or ε intronic promoters instead of the V_H promoter. Other variations of this plasmid excluded the hs4 enhancer.

Specific Aim 2 Results

Nomenclature

For the sake of clarity, the nomenclature used to describe the new *3'IghRR* reporter plasmids will follow a specific format. The name of each reporter will include the type of promoter in the plasmid as well as a reference to how much of the *3'IghRR* is included in the plasmid. For example, the reporter plasmid that includes sequence starting at the hs3 enhancer and ending at the hs1.2 enhancer (including the intervening sequence between those enhancers) and contains the V_H promoter is named pV_H.hs3-1.2 (Fig 11). If that plasmid contains the intronic ε promoter instead, it is named pIε.hs3-1.2. A plasmid that contains just the hs4 enhancer and the V_H promoter is named pV_H.hs4. Finally, a plasmid that contains the intronic γ_3 promoter, the hs4 enhancer, and the entire sequence from hs3 to hs1.2 is named pγ_3.hs3-1.2.4. Generally speaking, a dash between the enhancers means the sequence between the enhancers is included in the plasmid, but a period separating the enhancers means there is no intervening sequence between them.

The human 3'IghRR reporters pV_H.hs3-1.2 and pV_H.hs3-1.2.4 fail to respond to B-cell stimulation or TCDD

The human *3'IghRR* plasmids containing the intervening sequence between hs3 and hs1.2 failed to respond to B-cell stimulation or TCDD. It was expected that B-cell stimulation would increase the activity of the enhancers since B-cell stimulation is known to increase the transcriptional activity of the *IGH* gene, which is at least partially

driven by the *3'IghRR*. TCDD treatment was expected to result in reduced activity of the plasmids since TCDD is known to act as an immunosuppressant in both mouse and human B cells. However, when these plasmids were transfected into the human B cell line CL-01 and stimulated by either CD40L+IL-4 or by the TLR7/8 agonist R848 the reporters exhibited only a modest, but highly variable and statistically insignificant change in activity (Fig. 12 and (Alfaheeda, 2016)). Similarly, treatment of the transfected cells with TCDD, either in the stimulated or unstimulated state, also resulted in only a modest but not statistically significant response (Fig 12. and (Alfaheeda, 2016)). Variations in incubation time and treatment concentrations did not alter these trends. The V_H promoter in these plasmids was removed and the intronic ε and γ_3 promoters were inserted instead. Since class switch recombination involves transcription driven by the intronic promoters, and the *3'IghRR* helps drive that transcription, it was thought some difference in activity might be observed. A promoter was chosen from each of the two clusters of constant regions in case their positions are functionally relevant. Unfortunately, the results did not change regardless of the promoter used in the plasmid (Fig. 12 and (Alfaheeda, 2016)).

The human 3'IghRR reporter pV$_H$.hs4 fails to respond to B-cell stimulation but does respond to TCDD

In contrast to the reporters containing large sections of the *3'IghRR* sequence, the pV$_H$.hs4 reporter plasmid does respond to TCDD (Fig. 12 and (Alfaheeda, 2016)). Treatment of transfected CL-01 human B cells with TCDD slightly reduces activity of the

reporter. This result stands in stark contrast to a reporter containing the hs1.2 enhancer alone, which increased in activity with treatment of TCDD (Fernando et al., 2012; Freiwan, 2014). The conflicting outcomes between reporters containing either the hs1.2 enhancer or the hs4 enhancer might partially explain why the pV_H.hs3-1.2.4 plasmid did not respond to TCDD at all. However, the hs4 enhancer alone also failed to respond to B-cell stimulation, so the failure of the larger reporters to respond as predicted to either treatment probably cannot be explained entirely by the addition of hs4.

The human 3'IghRR reporter plasmids behave differently when transfected into human vs mouse B cells

It has been well established that reporter plasmids containing the mouse *3'IghRR* enhancers will, for the most part, exhibit exactly the type of activity one would expect when transfected into mouse cells and stimulated or treated with TCDD (Sulentic & Kaminski, 2011). That is to say, their activity increases in response to B-cell stimulation and decreases in response to TCDD, which is consistent with effects of B-cell stimulation and TCDD on both mouse and human B cell lines (Sulentic & Kaminski, 2011). Reporter plasmids bearing the human *3'IghRR* sequence, however, behave differently depending on if they are transfected into mouse or human cells. Previous work has shown that a plasmid containing just the human hs1.2 enhancer will show a decrease in activity in response to B-cell stimulation when transfected into human cells, whereas in mouse cells B-cell stimulation will increase its activity (Alfaheeda, 2016; Freiwan, 2014). TCDD will actually increase the activity of the human hs1.2 enhancer in both mouse and

human cells (Alfaheeda, 2016; Freiwan, 2014). When the human pV_H.hs3-1.2.4 plasmid is transfected into mouse cells, B-cell stimulation causes an increase in activity while TCDD causes a decrease. However, when the same plasmid is transfected into human B cells, it has little or no response to either treatment (Fig. 12 and (Alfaheeda, 2016)). It is unlikely this difference can be explained by a deficiency in the cell line being used since treatment with B-cell stimulation increases antibody secretion in CL-01 cells, and TCDD treatment decreases it, indicating the endogenous locus functions as expected. A more likely explanation is that some aspect of gene regulation by the human *3'IghRR* is not being captured by the human reporter plasmids, which means a method to study the endogenous *3'IghRR* is needed.

Mouse Reporter			Human Reporter			
Mouse Cells			Mouse Cells		Human Cells	
Stim	TCDD		Stim	TCDD	Stim	TCDD
↑	↓	hs1.2	↑	↑	↓	↑
—	↑	hs4	↑	↑	—	↓
↑	↓	Full Length	↑	↓	—	—

Figure 12. Summary of transfection studies using *3'IghRR* reporter plasmids. Arrows pointing up indicate an increase in reporter activity while arrows pointing down indicate a decrease in activity. A horizontal line indicates no significant change in activity. Mouse *3'IghRR* reporters typically show an increase in activity with B-cell stimulation and decrease in activity with TCDD treatment, except for the hs4 reporter. Human hs1.2 reporter plasmids, however, only show an increase in activity with B-cell stimulation when mouse B-cells are used. In human B cells, the human reporter decreases in activity with stimulation and increases activity with TCDD treatment. Transfection studies using the pGL3.hs3-1.2.4 (Full Length) plasmid, and its derivatives, exhibit similar activity to the mouse reporters when used in mouse cells, but show little change in activity when used in human B cells. For additional details about the human reporter plasmids refer to Zahra Alfaheeda's master's thesis (Alfaheeda, 2016).

IV. Specific Aim 3

Specific Aim 3 Rationale

As noted previously, there are pronounced structural differences between the human and mouse *3'IghRR*. The human *3'IghRR* is duplicated in humans, but not in mice, and the hs1.2 enhancer is polymorphic in humans but not in mice. The most obvious starting point for studying human *3'IghRR* function is to investigate the role of the *3'IghRR* duplication. Several distinct possibilities exist regarding the function of the *3'IghRR* duplication: 1) both the α_1 and α_2 regions may be required for successful transcription of any *IGH* isotype 2) the α_1 and α_2 regions may only regulate the transcription of their most proximal upstream constant regions 3) the α_2 region could be sufficient for production of any IGH isotype because the α_1 region will be lost during class switch recombination to certain isotypes 4) the duplication of the *3'IghRR* may be an entirely redundant and unnecessary by-product of evolution where either *3'IghRR* could be sufficient for production of any IGH isotype. The first hypothesis for specific aim 3 is that the α_1 *3'IghRR* mediates expression of the upstream constant regions while the second group of constant regions is controlled by the α_2 *3'IghRR*.

There are also modest structural differences between the human α_1 *3'IghRR* and α_2 *3'IghRR*, in particular in the hs1.2 enhancer. As previously described, this enhancer is polymorphic; it contains a 53 base pair invariant sequence (IS) that can be repeated up to four times in some individuals (Mills et al., 1997; Pinaud et al., 1997). This invariant sequence contains several transcription factor binding sites and increased numbers of repeats are associated with increasing enhancer activity in luciferase reporters (Denizot et al., 2001). The IS repeats within the α_1 hs1.2 enhancer have also been associated with

human disease states, which is strong evidence that these repeats are functionally significant (Aupetit et al., 2000; Cianci et al., 2008; Frezza et al., 2004; Frezza et al., 2009; Frezza et al., 2007; Tolusso et al., 2009; J. Wang & Boxer, 2005). The difference between the α_1 hs1.2 and α_2 hs1.2 enhancer is that the α_1 hs1.2 enhancer can have one, two, three, or four repeats of the IS, whereas the α_2 hs1.2 enhancer always contains either three or four repeats (Mills et al., 1997; Pinaud et al., 1997). Also, the two hs1.2 enhancers are in opposite orientation with respect to each other within the *3'IghRR* when a specific polymorphism is present (Denizot et al., 2001). Because the hs1.2 IS polymorphism does not exist in mice, all *3'IghRR* functional studies performed in mice have failed to elucidate the function of the IS polymorphism, which represents a tremendous gap in the literature in light of the association of the polymorphism with human diseases. Therefore, specific aim 3 will also test the hypothesis that specific combinations of the hs1.2 IS polymorphism will correspond to B cells preferentially expressing specific Ig isotypes.

Specific Aim 3 Results

Attempts to engineer a deletion of the α_1 3'IghRR failed

Attempts were made to delete the entire α_1 *3'IghRR* from the CL-01 human B cell line using two different CRISPR vectors (Fig. 13). This system takes advantage of a bacterial anti-virus defense mechanism which utilizes the Cas9 endonuclease to digest invading nucleic acids (Marraffini & Sontheimer, 2010). To make use of this mechanism in research, Cas9 has been inserted into a plasmid along with a guide RNA (gRNA) and

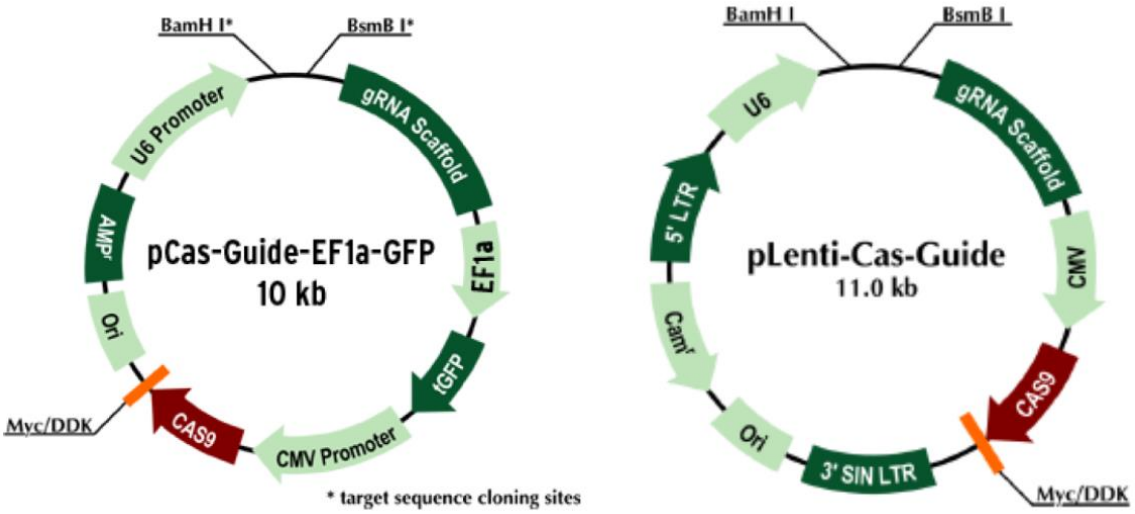

Figure 13. CRISPR plasmid maps. Two CRISPR plasmids were purchased from Origene (Rockville, MD) for use in this study. The first is a standard expression plasmid containing both the Cas9 endonuclease and the guide RNA (gRNA). This plasmid also expresses GFP. The second plasmid is a lentiviral vector which also contains the gRNA and Cas9, but does not express GFP. Definitions of abbreviations: U6 and CMV are promoters, AMP is an ampicillin resistance gene, CAM is a chloramphenicol resistance gene, EF1a is a promoter, GFP is green fluorescent protein, Ori is the origin of replication, Myc/DDK represents a Myc protein tag on the Cas9 endonuclease, 5'LTR stands for long terminal repeats and serves as a retroviral promoter, 3' SIN LTR stands for self-inactivating long terminal repeat and is a safety feature of many lentiviral plasmids.

a roughly 20 base pair targeting sequence (Mali et al., 2013). When the CRISPR plasmid is inserted into a cell, Cas9 protein is expressed and associates with the gRNA which is tethered to the targeting sequence (Deltcheva et al., 2011). When the targeting sequence base pairs with cellular DNA the Cas9 enzyme is pulled into proximity of the target site by the gRNA and creates a double-strand break (DSB) in the DNA (Deltcheva et al., 2011; Jinek et al., 2012). This double-strand break will then be ligated by one of two cellular processes, either non-homologous end-joining (NHEJ) or homology-directed repair (HR) (Mali et al., 2013). Exogenous DNA can be inserted into the target site by taking advantage of the homology directed repair machinery (Zheng et al., 2014). Alternatively, large genetic deletions can be created by introducing two double strand breaks via CRISPR and relying on non-homologous end joining to ligate the two breaks thereby excising the intervening sequence (Zhang et al., 2015). Non-homologous end joining is a highly error prone pathway which will often create small insertion or deletion mutations (indel). In some circumstances it may be desirable to use just one double strand break and rely on these indel mutations to disrupt small regions of the genetic code (Mali et al., 2013). This system has been used to reliably create genetic edits, including large deletions, in a wide array of species and cell types with a minimum of off target effects (He et al., 2015; Hilton et al., 2015; Li et al., 2015; Mali et al., 2013; Zhang et al., 2015; Zheng et al., 2014).

In order to make the deletion of the *3'IGHRR*, a double strand break needed to be introduced on either side of the α_1 *3'IghRR* using two different targeting sequences, one for the 5' end of the region and one for the 3' end (Table 2). This means that a cell

would need to be double transfected with two CRISPR vectors (one containing each targeting sequence) in order for the two double strand breaks to be induced. However, transfection efficiency in the CL-01 cells is very low (typically less than 10%) so the likelihood of any one cell being double transfected is also very low. Furthermore, deletions using this method tend to occur at frequencies of anywhere from 1-20%, so only a small population of cells would have the deletion out of an already small population of cells that was double transfected. The results reflected this difficult scenario as 105 clonal populations of cells transfected with the first set of CRISPR vectors were screened by PCR and found to be negative for the deletion (data not shown). A second set of CRISPR vectors, packaged into lentiviral particles and using the same targeting sequences, was also used. This second set of CRISPRs resulted in 189 clones being screened by PCR and they were also all negative for the deletion (data not shown). It is possible that the targeting sequences used for this attempted deletion were insufficient, but it could also be possible that low transfection/transduction efficiency was also a contributing factor. It might still be possible to make this deletion using different targeting sequences or by using two CRISPR vectors expressing different colored fluorescent proteins (for example, GFP and Texas Red) which would allow for sorting based on expression of both colors. For additional details on how this deletion was attempted refer to "Materials and Methods" of this dissertation.

The hs1.2 polymorphism was successfully edited using CRISPR

In contrast to the attempt at deleting the α_1 *3'IghRR*, editing the hs1.2 polymorphism using CRISPR worked with a surprisingly high rate of success. Because the hs1.2 polymorphism is a repeat of roughly 53 base pairs, it can be targeted multiple times with a single CRISPR vector, eliminating the need for a double transfection (Fig. 14 and 15). A CRISPR vector that targets the hs1.2 polymorphism and induces a double strand break in the NF1 transcription factor binding site resulted in positive edits in roughly 20% of the cells transfected with the CRISPR plasmid. A separate CRISPR plasmid that induced double strand breaks in the DRE binding site resulted in positive edits roughly 15% of the time. Using this method it was possible to create clonal cell populations which represent most of the possible combinations of the hs1.2 polymorphism (Fig. 16). Once these cell populations were derived they were examined for differences in antibody expression compared to unedited (wild type) CL-01 cells.

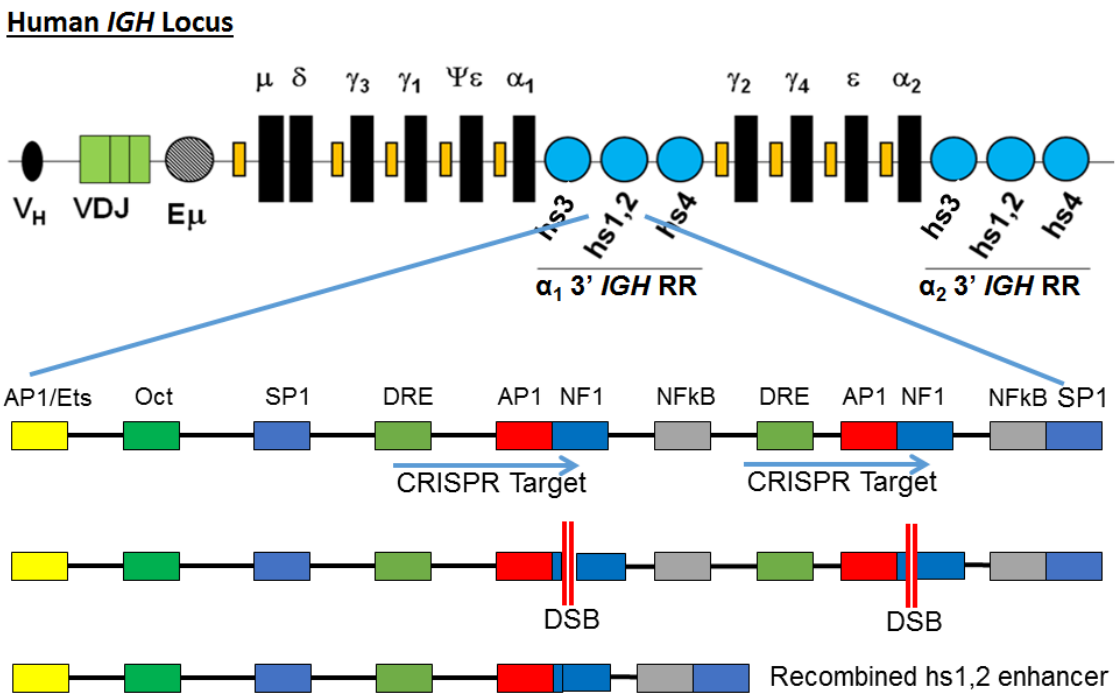

Figure 14. The α_1 hs1.2 enhancer targeted by CRISPR gene editing. The α_1 hs1.2 enhancer can be targeted twice by the same CRISPR targeting sequence that binds the α_2 hs1.2 due to the repetitive nature of the hs1.2 polymorphism. When the targeting sequence base pairs with the gene the Cas9 endonuclease induces a double strand break (DSB). The two DSBs can be ligated without the intervening sequence, effectively deleting an IS repeat, thus the α_1 hs1.2 enhancer can go from two repeats of the IS to one.

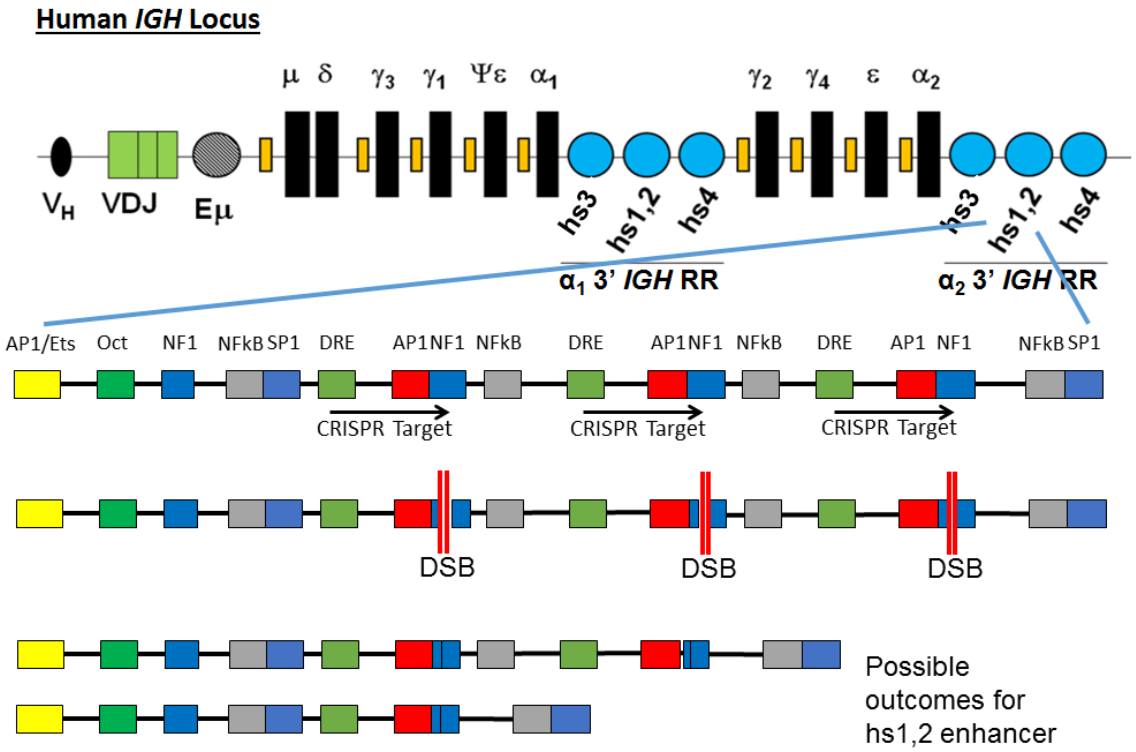

Figure 15. The α_2 hs1.2 enhancer targeted by CRISPR gene editing. The α_2 hs1.2 enhancer can be targeted three times by the same CRISPR targeting sequence that binds the α_1 hs1.2 due to the repetitive nature of the hs1.2 polymorphism. Due to some non-conserved sequence in the first IS, the α_2 hs1.2 in the CL-01 cells cannot be targeted four times in spite of containing four IS repeats. When the targeting sequence base pairs with the gene the Cas9 endonuclease induces a double strand break (DSB). The three DSBs can be ligated without one or both of the intervening sequences, thus the α_2 hs1.2 polymorphism can be reduced from four repeats to either three or two.

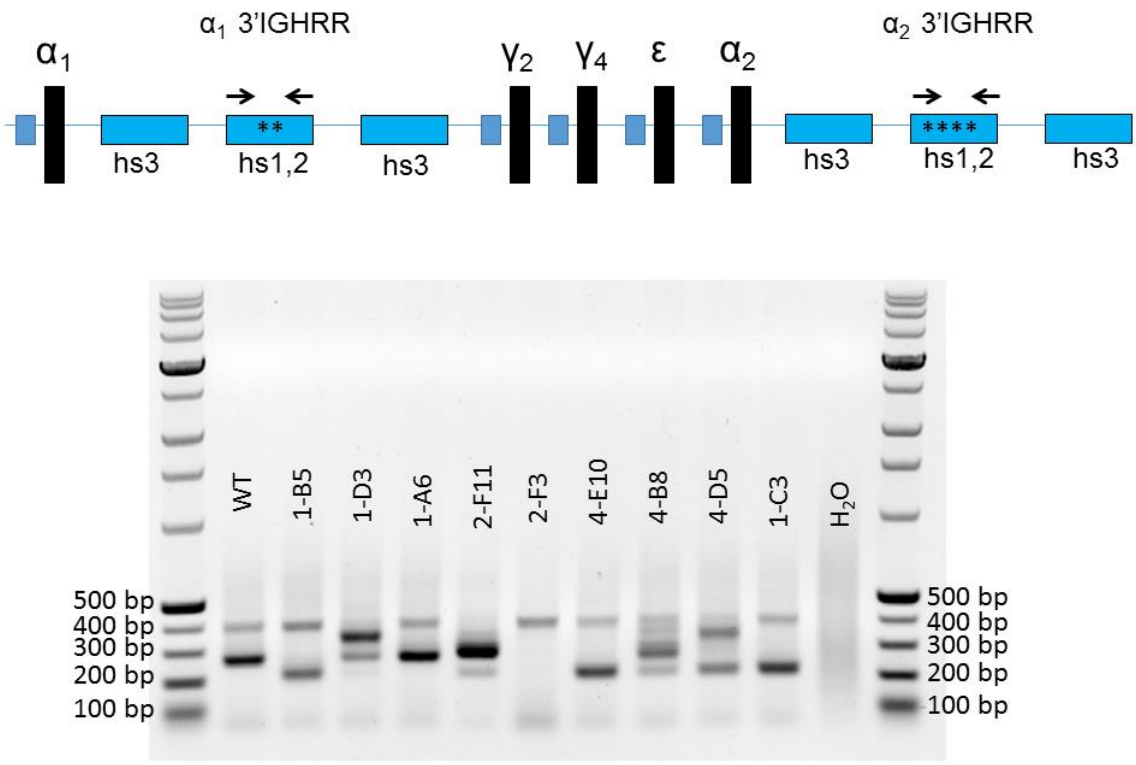

Figure 16. Genotyping the hs1.2 enhancer. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Wild type (WT) CL-01 cells show PCR products consistent with four IS repeats in the α_2 hs1.2 and two repeats in the α_1 hs1.2 enhancer. Clonal cell populations bearing numerous different combinations of the hs1.2 polymorphism were generated. Clone 1-C3 was lost due to user error, and clone 4-B8 has not been fully analyzed, so they are not included in this functional analysis.

Clone 1-D3 has three repeats of the IS in the α_2 hs1.2 and two repeats in the α_1 hs1.2

Based on PCR results that amplify both of the hs1.2 enhancers, clone 1-D3 has three repeats of the hs1.2 polymorphism in the α_2 hs1.2 enhancer and two repeats in the α_1 hs1.2. Cells were analyzed after stimulation with CD40 ligand and IL-4 followed by 96 hours of incubation. Analysis of secreted IgM by ELISA reveals that IgM secretion in this clone is unaffected by the change within the α_2 hs1.2 enhancer whereas IgG secretion is significantly reduced in both naïve and stimulated cells compared to wild type CL-01 cells (Fig. 17). Real time PCR analysis of Ig transcripts in clone 1-D3 stimulated with CD40L and IL-4 compared to stimulated wild type cells shows a slight increase in transcripts in most Ig isotypes, except γ_4 transcripts which are six fold higher in 1-D3 than in wild type cells. Since this large increase in γ_4 transcripts is not reflected in the secreted IgG ELISA data it is likely the increase in γ_4 transcripts is due to an increase in sterile germline transcripts. The α_1 and α_2 transcript levels are unchanged compared to wild type cells.

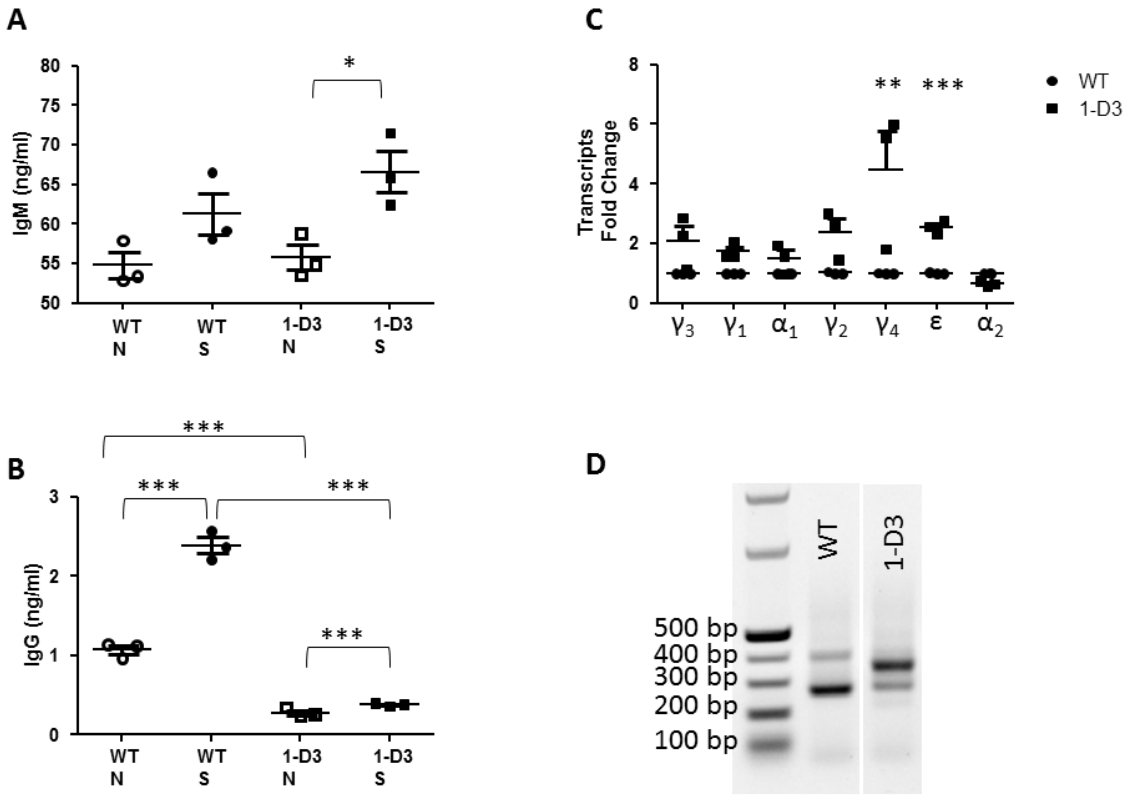

Figure 17. IGH expression in hs1.2-edited clone 1-D3. Wild type (WT) cells and clone 1-D3 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and secreted IgM (A) and IgG (B) were measured by ELISA. Transcription of the Ig constant regions were measured by real time PCR in stimulated cells and represented here in terms of fold change over stimulated WT cells (C). The genotype of the hs1.2 enhancers (D) in the modified clone versus wild type CL-01 cells demonstrates successful genetic editing. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

Clone 1-A6 appears to have the same combination of hs1.2 IS repeats as WT cells but expresses Ig isotypes differently

Clone 1-A6 appears to have four IS repeats in the α_2 hs1.2 enhancer and two IS repeats in the α_1 hs1.2 enhancer just like the WT cells. However, this clone behaves quite differently than WT cells (Fig. 18). Clone 1-A6 shows a modest reduction in secreted IgM and a large reduction in the amount of secreted IgG being expressed compared to WT cells. However, comparing the amount of γ transcripts of all subtypes in stimulated 1-A6 compared to stimulated WT cells reveals very little difference. The most significant change in transcript levels is in the two α subtypes and ε transcripts. Both the α_1 and α_2 isotypes show a significant reduction in transcript levels whereas the ε transcripts are 3.5 fold higher in clone 1-A6 than in WT cells. So, how can clone 1-A6 be expressing Ig differently than WT cells when they have the same genotype? The most likely explanation is that clone 1-A6 experienced a small insertion or deletion (indel) mutation when the CRISPR-induced double stand breaks were ligated through non-homologous end joining (Fig. 19). If this is true, it means all the differences Ig expression observed with this clone are the result of a disruption in the NF1 transcription factor binding site. Attempts to verify this hypothesis by sequencing the genotyping PCR products have so far met with limited success. One reliable sequence was generated for each product, but the sequence was identical to wild type cells. This does not mean 1-A6 is a wild type cell,

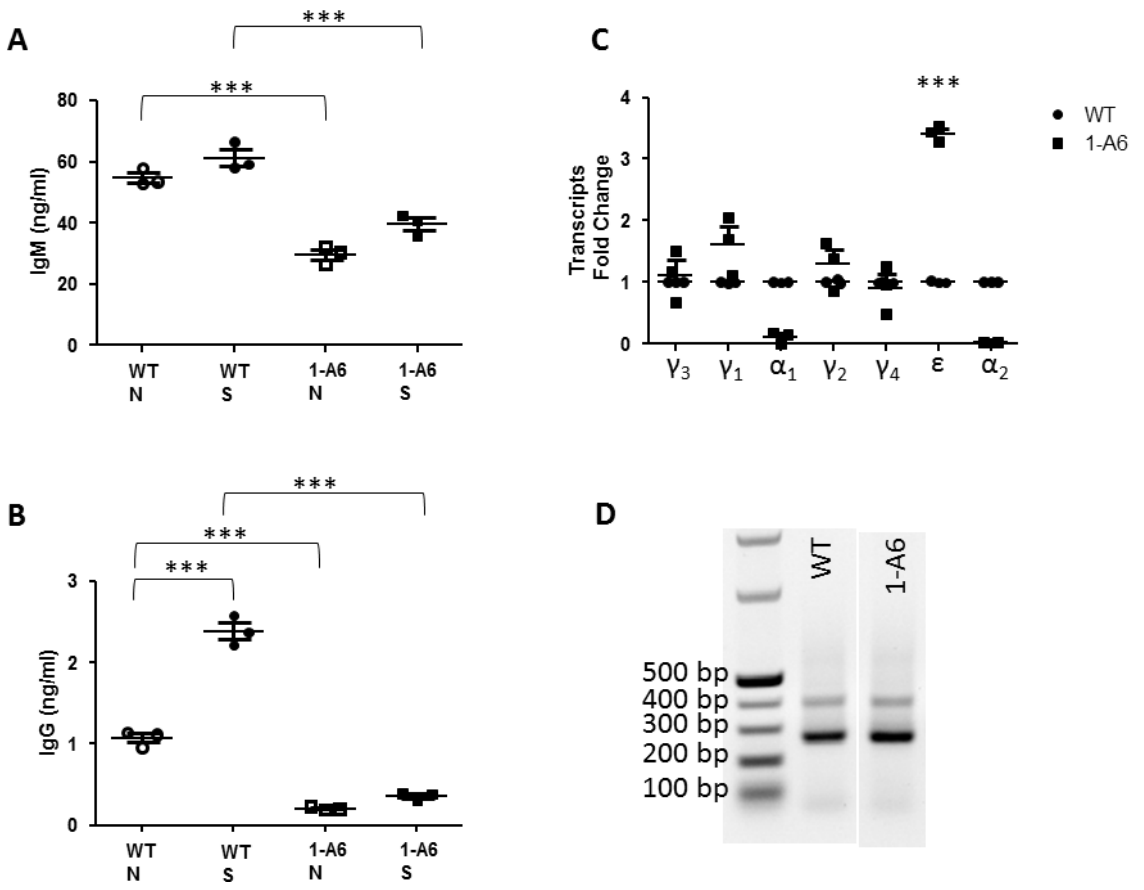

Figure 18. IGH expression in hs1.2-edited clone 1-A6. Wild type (WT) cells and clone 1-A6 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and secreted IgM (A) and IgG (B) were measured by ELISA. Transcription of the Ig constant regions were measured by real time PCR in stimulated cells and represented here in terms of fold change over stimulated WT cells (C). The genotype of the hs1.2 enhancers (D) in the modified clone versus wild type CL-01 cells appears identical on an agarose gel even though the clone has a different Ig expression profile. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

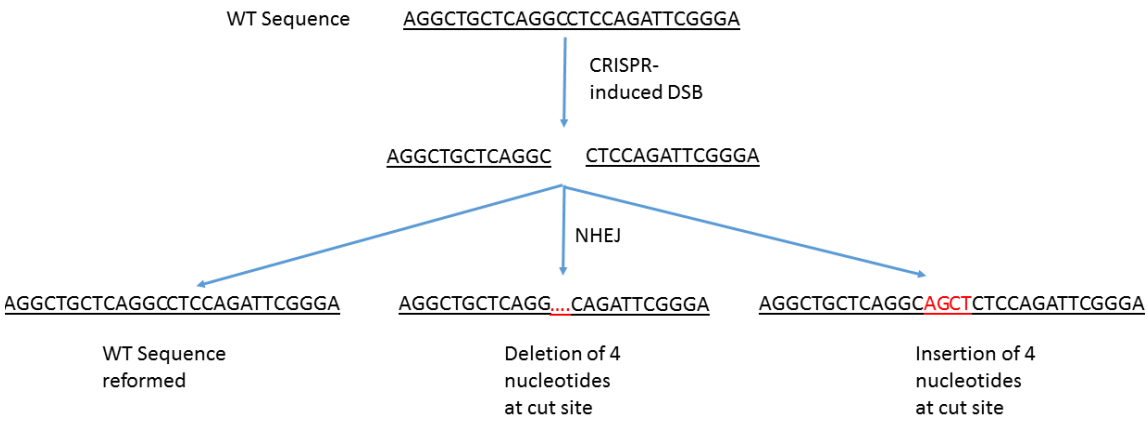

Figure 19. Non-homologous end joining (NHEJ) and lead to insertion/deletion (indel) mutations. If the CRISPR-induced double strand break is repaired by NHEJ a random number of nucleotides can be inserted or deleted from the DNA sequence because NHEJ is an error prone pathway.

however, because the sequencing data might represent only one allele due to the sequencing method employed. The other allele might have a mutation. But since it is currently unknown which allele was successfully sequenced (it could have been either the functional or translocated allele) it's impossible to precisely define the genotype of clone 1-A6.

Clones 1-B5 and 4-E10 have four IS repeats in the α_2 hs1.2 enhancer and one IS in the α_1 hs1.2

Although they have different genotypes clone 1-B5 behaves very similarly to clone 1-A6 in terms of Ig expression (Fig. 20). This clone has very slightly reduced expression of secreted IgM but dramatically reduced expression of secreted IgG. All four of the γ subtypes are showing elevated transcript levels, with roughly a 2 fold increase over WT cells. Similar to clone 1-A6, both α subtypes are reduced compared to WT cells, whereas the ε transcripts are 6 fold higher in the modified clone than in WT cells. The similarity in expression patterns between 1-A6 and 1-B5 might indicate that an indel mutation occurred in the α_1 hs1.2 enhancer of clone 1-A6 that disrupted a transcription factor binding site essential for normal expression of α and ε transcripts. It would be expected that a clone with the same genotype as 1-B5 would have the same pattern of Ig expression, but clone 4-E10 expressed Ig isotypes differently than 1-B5 despite having the same genotype (Fig. 21). Secreted IgM and IgG are expressed in a similar way in 4-E10 as in 1-B5, namely, both isotypes are secreted at lower levels than in WT cells. However, transcript levels in stimulated 4-E10 are not significantly different than in

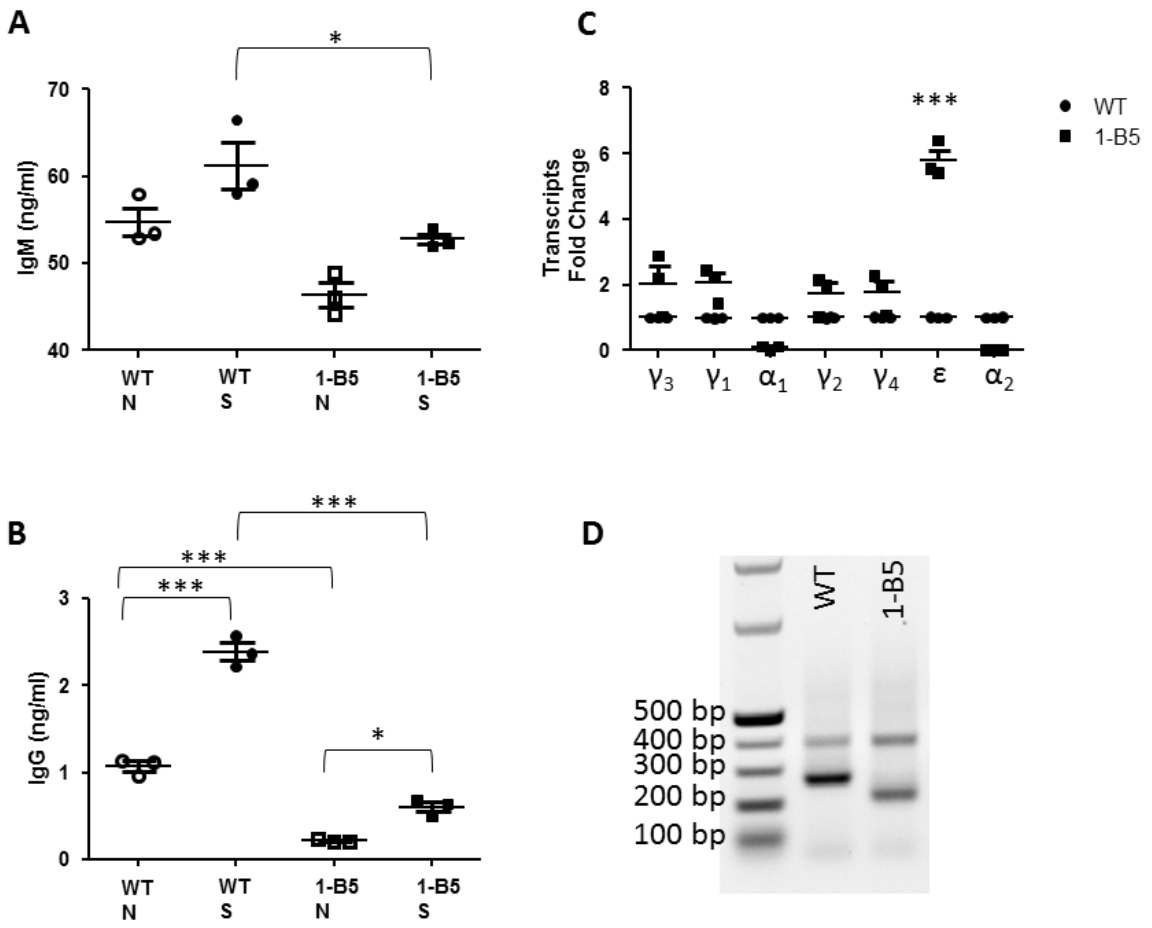

Figure 20. IGH expression in hs1.2-edited clone 1-B5. Wild type (WT) cells and clone 1-B5 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and secreted IgM (A) and IgG (B) were measured by ELISA. Transcription of the Ig constant regions were measured by real time PCR in stimulated cells and represented here in terms of fold change over stimulated WT cells (C). The genotype of the hs1.2 enhancers (D) in the modified clone versus wild type CL-01 cells demonstrates successful genetic editing. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

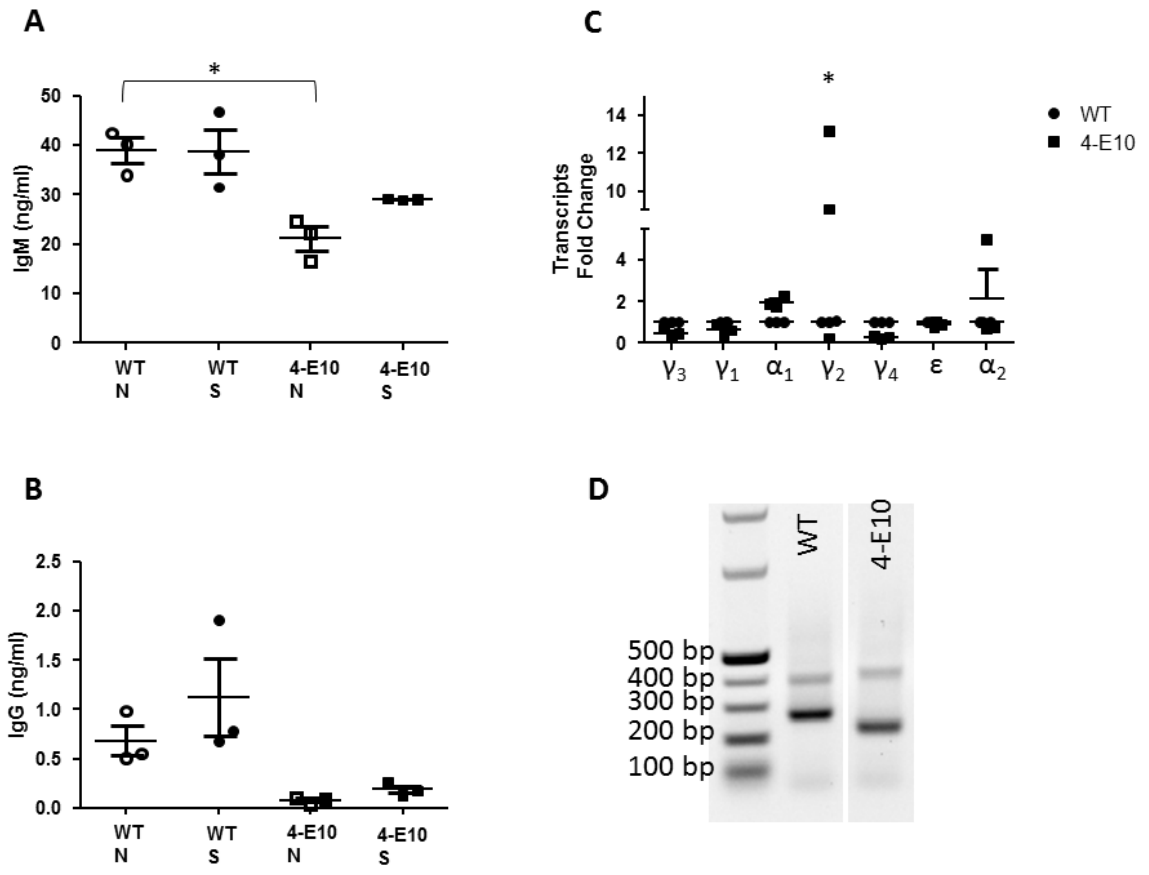

Figure 21. IGH expression in hs1.2-edited clone 4-E10. Wild type (WT) cells and clone 4-E10 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and secreted IgM (A) and IgG (B) were measured by ELISA. Transcription of the Ig constant regions were measured by real time PCR in stimulated cells and represented here in terms of fold change over stimulated WT cells (C). The genotype of the hs1.2 enhancers (D) in the modified clone versus wild type CL-01 cells demonstrates successful genetic editing. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

stimulated WTcells for all Ig isotypes except for γ_2 transcripts. The difference in Ig expression patterns between 4-E10 and 1-B5, despite them both having the same genotype, could be due to the presence of small indel mutations that are undetectable on an agarose gel. Thorough sequencing of both clones will be necessary to determine if significant genetic differences exist between the clones that might explain these differences.

Clone 2-F11 has a genotype that was not predicted

Clone 2-F11 is unusual because it shows a genotype that was not predicted prior to the isolation of clonal populations transfected with the CRISPR plasmid. The genotyping PCR produces a band that runs halfway between the expected sizes of a two IS repeat and three IS repeat of the hs1.2 enhancer (Fig. 16 and 22). A second band runs at roughly the predicted size for one IS. The genotyping PCR typically produces a much thicker, darker band for this clone than for any of the other clones and it is always the abnormally sized band that is darker. Attempts to sequence that PCR product have so far been unsuccessful. Clone 2-F11 produces secreted IgM at roughly equivalent levels as WT cells (Fig. 22). Secreted IgG in naïve 2-F11 cells and WT cells are very similar, but secreted IgG levels do not increase with stimulation as much for 2-F11 as they do for stimulated WT cells. This stands in stark contrast to γ transcripts, which are all elevated in stimulated 2-F11 compared to stimulated WT cells. This probably reflects an increase in germline transcription in 2-F11. Transcripts of ε are elevated by roughly 2 fold over WT cells, whereas transcription of both α subtypes is reduced.

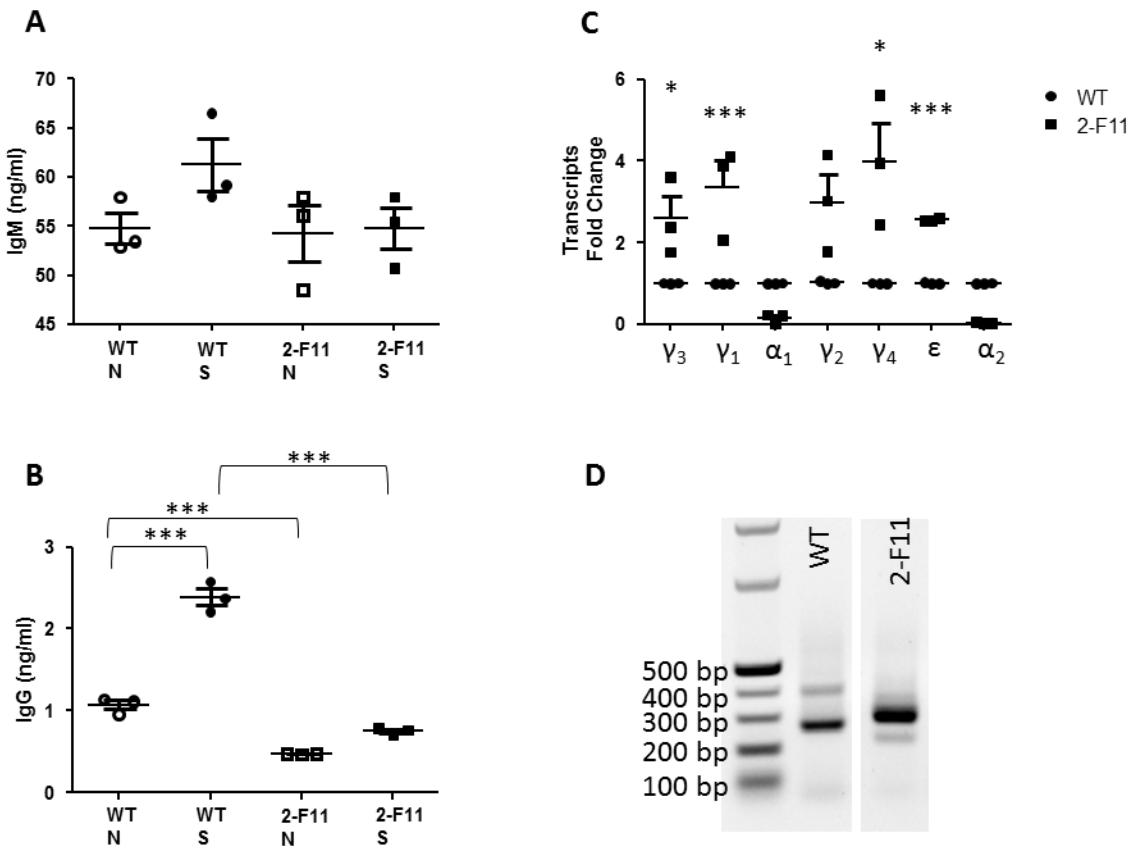

Figure 22. IGH expression in hs1.2-edited clone 2-F11. Wild type (WT) cells and clone 2-F11 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and secreted IgM (A) and IgG (B) were measured by ELISA. Transcription of the Ig constant regions were measured by real time PCR in stimulated cells and represented here in terms of fold change over stimulated WT cells (C). The genotype of the hs1.2 enhancers (D) in the modified clone versus wild type CL-01 cells demonstrates successful genetic editing. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

Both of the hs1.2 enhancers have been edited in clone 4-D5

Clone 4-D5 is the only clone produced that has had both of the hs1.2 enhancers edited. All of the other clones have at least one enhancer that appears to be unedited, although small indel mutations cannot be ruled out at this time. Clone 4-D5 has three IS repeats in the α_2 hs1.2 enhancer and one IS in the α_1 hs1.2. This clone exhibits a significant reduction in secreted IgM as well as IgG (Fig. 23). It's also the only clone in which transcripts for all the Ig isotypes are expressed at lower levels than WT when the cells are stimulated. The α_1 and γ_2 transcripts see the biggest reductions compared to WT cells while γ_1 and ε transcripts are more modestly reduced.

The α_1 hs1.2 enhancer appears to have been entirely deleted in clone 2-F3

Clone 2-F3 is unique in that it appears to have the entire α_1 hs1.2 deleted based on the results of the genotyping PCR (Fig. 16 and 24). The α_2 hs1.2 appears to be unedited in this clone. When a double strand break is repaired by the homology directed repair mechanism in the absence of donor DNA with homologous sequence near the break a 5' resection can occur that results in some portion of the genome being deleted (Fig. 25) (Paudyal & You, 2016). It is likely this mechanism has deleted some portion of the α_1 hs1.2 enhancer, but it is unknown how much sequence was lost. Interestingly, despite the apparent loss of the α_1 hs1.2 enhancer, secreted IgM and IgG appear to have been mostly unaffected by this genetic edit (Fig. 24). Examination of Ig transcripts, however, reveals that several of the Ig isotypes are being transcribed at lower levels in stimulated 2-F3 then in stimulated WT cells. Of special significance is a

large reduction in ε transcription which suggests the α_1 hs1.2 enhancer is surprisingly important in the expression of the downstream cluster of constant regions.

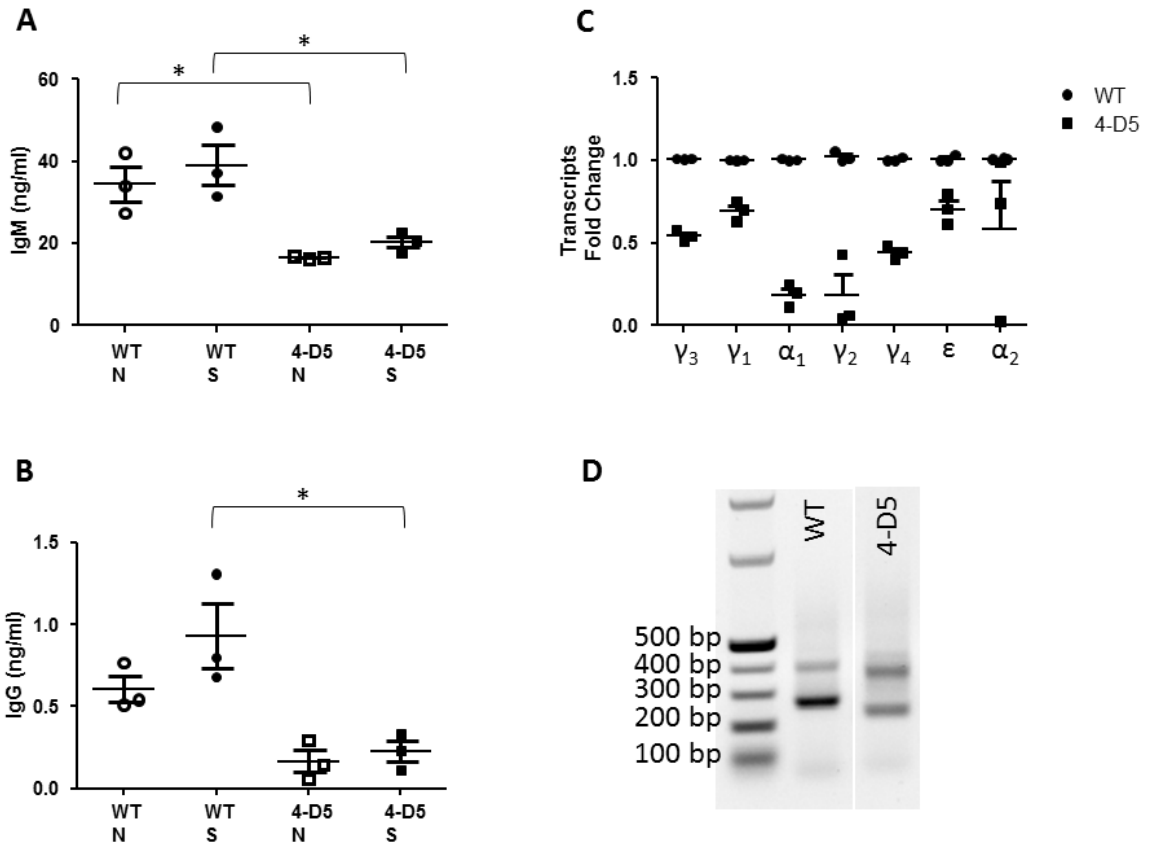

Figure 23. IGH expression in hs1.2-edited clone 4-D5. Wild type (WT) cells and clone 4-D5 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and secreted IgM (A) and IgG (B) were measured by ELISA. Transcription of the Ig constant regions were measured by real time PCR in stimulated cells and represented here in terms of fold change over stimulated WT cells (C). The genotype of the hs1.2 enhancers (D) in the modified clone versus wild type CL-01 cells demonstrates successful genetic editing. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

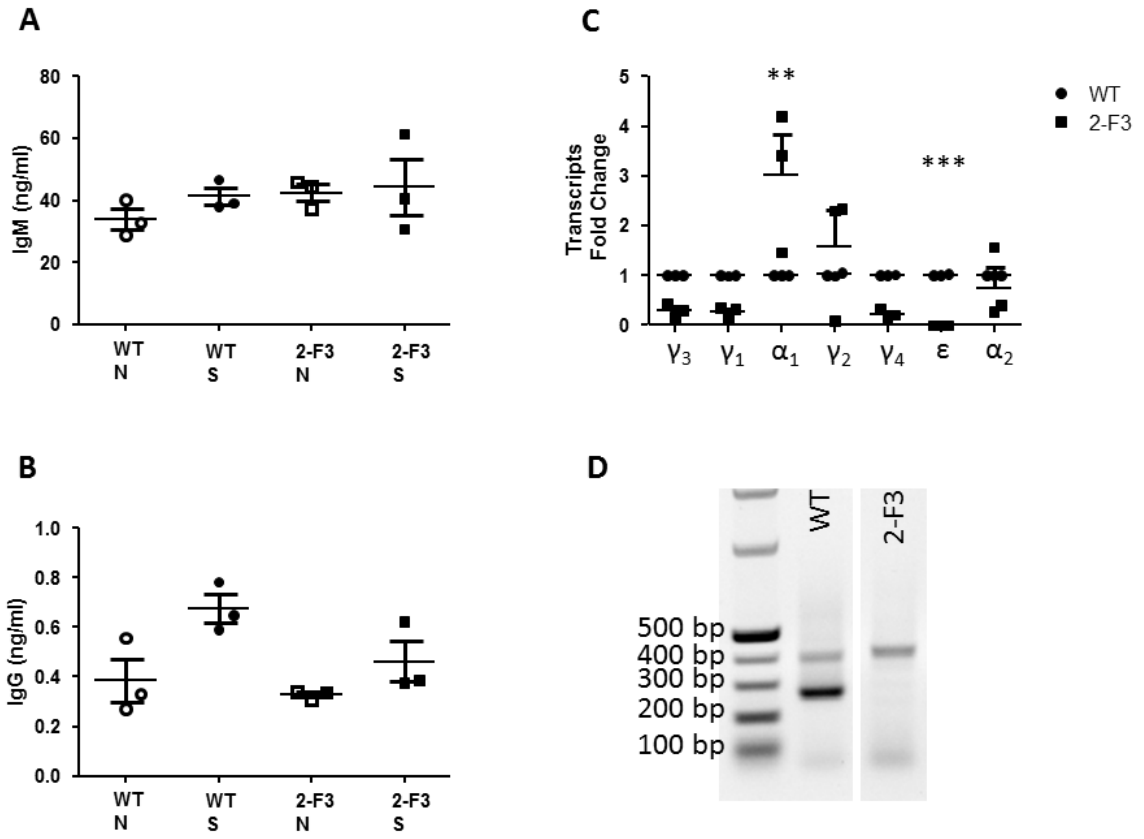

Figure 24. IGH expression in hs1.2-edited clone 2-F3. Wild type (WT) cells and clone 2-F3 were stimulated with CD40L (6.25 ng/ml) and IL-4 (25 ng/ml) and secreted IgM (A) and IgG (B) were measured by ELISA. Transcription of the Ig constant regions were measured by real time PCR in stimulated cells and represented here in terms of fold change over stimulated WT cells (C). The genotype of the hs1.2 enhancers (D) in the modified clone versus wild type CL-01 cells demonstrates successful genetic editing. Due to complete sequence homology between the α_1 hs1.2 and α_2 hs1.2 enhancers outside of the polymorphism, a single pair of PCR primers can be used to amplify both enhancers at the same time. Predicted product sizes are 374 bp for four IS repeats, 319 bp for three IS repeats, 264 bp for two repeats, and 209 bp for one IS. Comparisons between treatment groups were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post-test. One, two, or three asterisks (*) denote significance compared to the corresponding control at $p<0.05$, $p<0.01$, or $p<0.001$ respectively.

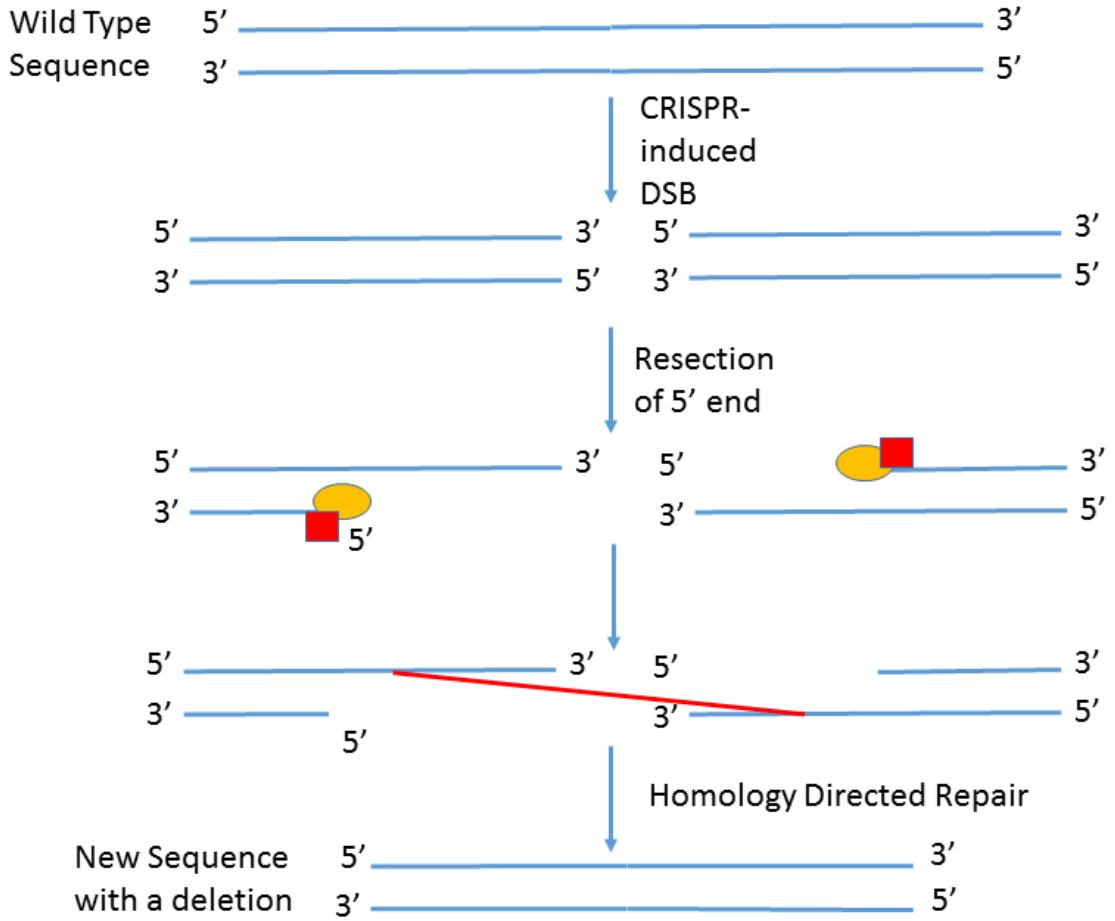

Figure 25. 5′ resections can lead to large genomic deletions. If a cell attempts to repair a CRISPR-induced double strand break using the homology directed repair mechanism without homologous donor DNA present, a 5′ resection can occur. When this happens, the 5′ end of the double strand break is degraded until enough homologous DNA is found on the 3′ overhang for homology directed repair to seal the break.

V. Discussion

Our previous work has shown the mouse 3' IghRR to be a sensitive target of TCDD, and further work with an AhR antagonist or shRNA knockdown of the AhR strongly support an AhR-mediated mechanism for the effects of TCDD (Sulentic et al., 1998, 2000; Sulentic et al., 2004; Wourms & Sulentic, 2015). However, it is unknown exactly where in the human 3' IGHRR AhR might be binding or if instead of directly binding to DNA AhR might be interacting with, or altering expression of, other transcription factors that bind to the 3' IGHRR. Out of the three enhancers found in the human *3'IghRR*, the hs1.2 enhancer has the highest density of transcription factor binding sites, and is the only one known to be polymorphic in the human population (Sulentic & Kaminski, 2011). All four versions of the polymorphic hs1.2 enhancer are sensitive targets of TCDD, and therefore could be sensitive to other exogenous AhR ligands, making the hs1.2 enhancer a potential target of immunotherapies. These polymorphisms have been associated with a variety of immunological disorders including lupus erythematosus, dermtatis herpatiformis, plaque psoriasis, psoriatic arthritis, rheumatoid arthritis, systemic scerlosis, coeliac disease, IgA deficiency, IgA nephropathy, and even schizophrenia and AIDS progression so the hs1.2 enhancer is of particular interest to human health (Aupetit et al., 2000; Cianci et al., 2008; Frezza et al., 2004; Frezza et al., 2009; Frezza et al., 2007; Frezza et al., 2012; Giambra et al., 2009; Montesano et al., 2014; Tolusso et al., 2009). Therefore, one goal of this project was to evaluate the individual contributions of each transcription factor binding site to hs1.2 enhancer activity.

Assessing the role of the AP1.Ets binding site through site-directed mutagenesis of the hs1.2 luciferase reporter has revealed this site to be a strong regulator of overall hs1.2 enhancer activity. In stark contrast to every other binding site mutation assessed in this study, the AP1.Ets site is the only one that reduced activity of the hs1.2 enhancer. This result suggests the AP1.Ets site is a strong positive regulator of hs1.2 enhancer activity. Other studies have shown c-Jun and c-fos to be the transcription factors that bind to this enhancer, but it would be useful to know if any other proteins could bind to this site (Sulentic & Kaminski, 2011). Because the hs1.2 enhancer is associated with so many human diseases, and because the AP1.Ets site has now been shown to be a strong regulator of hs1.2 enhancer activity, a possibility exists that this binding site could be a therapeutic target. For example, in lymphoma patients with chromosomal translocations of the 3' IGHRR to proto-oncogenes (i.e. c-myc, bcl-2, ect), blocking this site might significantly reduce the potential oncogenic effect of the translocation.

Like the AP1.Ets site the transcription factor, octamer is located 5' of the invariant sequence. Oct contributes to mouse hs1.2 enhancer activity and is conserved between mouse and human (Mills et al., 1997). In B cells, Oct in collaboration with G-rich, κB-like motifs and Pax5 repress transcription of the murine hs1.2 enhancer (Singh & Birshtein, 1996). Our mutation of the Oct binding site in the human hs1.2 enhancer suggests a conservation of function in the human locus, since mutating the Oct binding site lead to an increase in overall luciferase activity. Furthermore, an Oct binding motif is frequently present in AhR-sensitive genes, suggesting a potential role in AhR signaling and TCDD-induced modulation (Kel et al., 2004). In the current study, mutation of the

Oct binding site in the hs1.2 reporter plasmid significantly increased TCDD-induced activation in unstimulated B cells. Under these conditions, Oct must act as an inhibitor of TCDD-induced activity. However, this effect was not observed in LPS-stimulated B cells, perhaps some aspect of TLR-4 signaling masks or eliminates the inhibitory effect of Oct on TCDD-induced hs1.2 activation. Additionally, cellular stimulation increases AhR expression (Sulentic et al., 2000), so perhaps the additional AhR protein can compensate for the inhibitory effect of Oct (C. F. Vogel et al., 2014). Another possibility is that TLR-4 signaling can lead to increased expression of transcription factors that target the hs1.2 enhancer, such as NF-κB and AP-1, and these proteins could be overriding an inhibitory role of Oct in TCDD-induced activation when B cells are stimulated (C. F. Vogel et al., 2014).

A number of studies have reported a TCDD-induced influence on AP-1, but outcomes vary on whether the transcription factor's activity increases or decreases. In LPS-activated CH12.LX cells, one study showed that TCDD up-regulated AP-1 binding within the promoter of B lymphocyte–induced maturation protein-1 (Blimp-1), a critical regulator of B-cell differentiation and a negative transcriptional repressor of Pax5 (Schneider, Manzan, Yoo, Crawford, & Kaminski, 2009). In this same study a link was made between TCDD-mediated suppression of Blimp-1 through AP-1 binding and Pax5 dysregulation (Schneider et al., 2009). Also, other studies conducted in multiple liver cell types showed an increase in AP-1 DNA binding activity resulting from genes induced by AhR agonists (Ashida, Nagy, & Matsumura, 2000; Puga, Nebert, & Carrier, 1992). In opposition Suh and colleagues demonstrated that TCDD inhibited DNA binding and

transcriptional activity of AP-1 in LPS-activated CH12.LX cells (Suh et al., 2002). In the same study TCDD was unable to inhibit AP-1 activity in an AhR- deficient murine B cell line, BCL-1 (Suh et al., 2002). Even though TCDD-induced effects on AP-1 differ, it is clear that AP-1 effected by TCDD. In this study, no significant effect on TCDD-induced activity of the human hs1.2 enhancer was observed, although the α_{1A}AP1mut resulted in significantly higher transcription by the enhancer in both unstimulated and LPS-stimulated CH12.LX cells. The AP-1 site seems to play an inhibitory role in the overall transcription of the human hs1.2 enhancer, but doesn't appear to play a role in TCDD-induced modulation, a surprising result in the context of the previous work. If TCDD truly inhibits AP-1 binding in CH12.LX cells, our reporter system would not likely detect a TCDD effect on the already mutated AP-1 binding site, so this study does not rule out the possibility of a TCDD/AhR-AP-1 interaction.

Like the IS AP-1 site, TCDD-induced modulation of the human polymorphic hs1.2 enhancer was thought to involve the IS NF-κB site. NF-κB activity contributes to the activation of mouse hs1.2 enhancer at the plasma cell stage (Michaelson et al., 1996). Also, NF-κB has been found to modulate AhR signaling, which may explain how TCDD-induced immunotoxicity is mediated (Tian, 2009; C. F. Vogel et al., 2014). Transcriptional activity of the AhR and NF-κB has been shown to be regulated by the same co-repressors and co-activators, such as steroid receptor coactivator-1 (SRC-1) and p300/CBP (Tian, 2009). Because co-regulators are required for both pathways, it is possible that competition for binding occurs causing one pathway to be active while the other pathway is suppressed (Tian, 2009). Once again, the results of our mutational

analysis indicate that binding of the NF-kB transcription factor to its putative binding site does not affect TCDD-induced activity of the hs1.2 enhancer. However, transient transfections of CH12.LX cells resulted in significantly higher transcriptional activity from mutation of the NF-κB site in both unstimulated and LPS-stimulated cells.

This mutational analysis of the human hs1.2 enhancer's transcription factor binding sites has yielded some surprising results. The DRE is often assumed to be a binding site of the AhR due to work in the CYP1A1 locus and this was thought to be the case in the hs1.2 enhancer as well (reviewed in (Beischlag, Luis Morales, Hollingshead, & Perdew, 2008; Hanieh, 2014; Quintana, 2013)). In fact, our previous work has shown that AhR binds to the DRE in the mouse hs1.2 enhancer (Salisbury & Sulentic, 2015; Sulentic et al., 1998, 2000; Sulentic et al., 2004). However, the results of our mutational analysis suggest that either the AhR is not binding to the DRE in the human hs1.2 or that such binding is unnecessary for AhR-mediated induction of hs1.2 activity. Instead, the nuclear factor 1 transcription factor binding site appears to be a much more important regulator of TCDD-induced activity of the hs1.2 enhancer. It is not immediately clear why this might be the case. Perhaps the AhR is binding to NF1 through protein-protein interactions rather than binding directly to the DNA. There is evidence in the literature to suggest that the AhR can bind directly to other transcription factors (Kobayashi et al., 1996; Tian, 2009; F. Wang et al., 1999), but so far no one has shown a direct interaction between AhR and NF1. Another possibility could be a cooperative interaction involving AhR, NF1, SP-1, Oct, and NF-kB since AhR binding to SP-1 and NF-kB has been demonstrated previously as well as an interaction between NF1 and SP-1 (Kobayashi et

al., 1996; Sepulveda, Emelyanov, & Birshtein, 2004; Tian, 2009; C. F. Vogel et al., 2014; F. Wang et al., 1999). A complicating factor is that our NF1 binding site mutation only showed reduced TCDD-induced activity when the cells were stimulated with LPS. This suggests that some aspect of TLR-4 signaling or B-cell stimulation is required. The specific mechanism that triggers this potential association is unknown, but it might be related to the increased expression of AhR following LPS stimulation (C. F. Vogel et al., 2014). Furthermore, since mutation of the NF1 binding site only reduces, but does not eliminate TCDD-induced activation of the hs1.2 enhancer there must be an additional unknown mechanism at work. Regardless, the results of this study indicate that TCDD-induced activity of the hs1.2 enhancer occurs through a non-canonical AhR mediated pathway involving Oct and NF1.

This is the first study to attempt to understand the individual contributions of each transcription factor binding site within the hs1.2 enhancer of the 3' IghRR. Understanding the regulatory framework which modulates IgH expression is vital to finding new treatments for immunological diseases and the pathologies associated with exogenous chemicals that affect the immune system. Our findings indicate the hs1.2 enhancer is subject to a complex regulatory scheme involving many transcription factors that might bind directly to the enhancer itself or alter activity by binding to each other. Different types of B-cell stimulation are likely to play a key role in how these transcription factors carry out their functions since different stimulatory pathways will likely result in a slightly different mix of factors playing out their roles within the hs1.2 enhancer. Further studies involving additional types of B-cell stimulation are needed to

fully understand when and why each transcription factor binding site comes into play. Furthermore, the hs1.2 enhancer might interact with the IgH intronic promoters as well as the VH promoter, so our study might have missed significant effects of some mutations, especially if certain hs1.2 transcription factors only function in the context of specific promoters. Therefore, future studies should also be performed using alternative promoters. However, this study provides a rough outline of the relative effect of each transcription factor binding site within the hs1.2 enhancer, which is a significant first step in understanding the complexity of IgH regulation.

As work was underway to understand the activity of the human hs1.2 enhancer something strange was noticed about the activity of the human hs1.2 reporter plasmid. When the human hs1.2 enhancer was transfected into human B cells, which were then stimulated by CD40L and IL-4 (or other types of stimulation) the activity of the plasmid goes down compared to unstimulated cells. This response to stimulation is the opposite of what was expected and it is the opposite of what the mouse hs1.2 reporter does in mouse B cells. When a B cell is subject to stimulation the *3'IghRR* is expected to drive an increasing amount of Ig expression so reporter plasmids containing elements of the *3'IghRR* would also be expected to increase their activity in response to B-cell stimulation. These expectations have been reinforced by numerous reporter plasmid studies using elements of the mouse *3'IghRR*. So, the fact that the human hs1.2 reporter plasmid decreases in activity when transfected into stimulated human B cells was an unexpected and puzzling finding. The picture was further complicated when it was noticed that transfecting mouse B cells with the human hs1.2 reporter plasmid results in

an increase in activity with B-cell stimulation, just as was expected with human cells. Unfortunately, treating cells with TCDD after transfection with the human hs1.2 reporter resulted in further confusion.

Since TCDD is known for its immunosuppressive effects in animal models it was expected that treating human B cells with TCDD would result in lower expression of Ig, which is exactly what happens in the CL-01 human B cell line when secreted IgG is measured by ELISA. Logically, the human hs1.2 reporter plasmid would be expected to show a decrease in activity when transfected into human B cells treated with TCDD since the hs1.2 enhancer is thought to be partially driving transcription in the endogenous locus. However, the opposite is observed. Treating either human or mouse B cells with TCDD and transfecting them with the human hs1.2 reporter plasmid results in an increase in activity. In contrast, the mouse hs1.2 reporter plasmid does show the expected decrease in activity when transfected into mouse B cells and treated with TCDD. Taken together, the surprising results from transfection studies using the human hs1.2 reporter plasmid, and its unexpected response to B-cell stimulation and TCDD, suggests that some essential aspect of *3'IghRR* activity is being missed when the human hs1.2 enhancer is studied in isolation. It's possible that removing the human hs1.2 enhancer from the context of the endogenous *3'IghRR* results some alteration to the way in which its activity would normally be regulated. Therefore, putting the human hs1.2 enhancer back into a more natural context, with additional elements of the *3'IghRR*, might restore the activity of the human reporter plasmid to something more closely resembling results from the mouse reporter studies.

With all of this in mind, a new human *3'IghRR* reporter plasmid has been constructed that includes more of the *3'IghRR* sequence to put the hs1.2 enhancer into a context more similar to the endogenous locus. The new reporter includes sequence that starts just 5' of the hs3 enhancer and runs all the way through the hs1.2 enhancer, including all the sequence between these two enhancers. It has long been thought that the sequence between the enhancers of the *3'IghRR* has some functional significance. The intervening sequences include long stretches of nucleotide repeats, which might be considered microsatellite repeats, and are thought to contribute to the formation of secondary structures in the *3'IghRR*. Recent work using knock out mice in which these sequences were deleted revealed a steep decrease in Ig expression in their absence (Garot et al., 2016). By including the 5 kb of sequence between hs3 and hs1.2 it was hoped that some of these secondary structures might be preserved in the new reporter plasmid. Unfortunately, due to the technical difficulty involved the roughly 15 kb of sequence between hs1.2 and hs4 could not also be included in the reporter. Additional nucleotide repeats are present in that portion of the sequence, which means that essential secondary structures could still be missed in studies using the new reporter. Another interesting feature of the intervening sequence between the *3'IghRR* enhancers is the presence of many potential nuclear factor binding sites. These sites match the consensus binding sequence for AP-1 and DRE, but it's not known if these sites are functional or not. There are three potential AP-1 sites and three potential DRE sites in the sequence between hs3 and hs1.2, which was included in the new plasmid.

Additional potential binding sites are present in the sequence between hs1.2 and hs4, which was not included in the plasmid.

Several different versions of the new *3'IghRR* reporter plasmid have been constructed. One version of the plasmid (pV_H.hs3-1.2) includes the sequence from hs3 all the way to hs1.2 but does not include the hs4 enhancer. A second version of the plasmid (pV_H.hs3-1.2.4) is identical to the first but has the hs4 enhancer ligated to the 3' end of the hs1.2 enhancer. Both of these versions of the plasmid are under the control of the variable heavy chain promoter (V_H). It's thought that the *3'IghRR* can also interact with the Ig heavy chain intronic promoters. These promoters sit 5' of each Ig constant region and become active during class switch recombination. They drive the transcription of sterile, germline transcripts which are not translated into protein (Ju et al., 2011; Ju et al., 2007). Since one possible explanation for the unexpected outcomes with the human hs1,2 reporter plasmid could be the use of the V_H promoter rather than an intronic promoter, some of these intronic promoters were included in versions of the new reporter plasmid. The γ_3 intronic promoter was included in the plasmid designated $p\gamma_3$.hs3-1.2.4 and the ε intronic promoter was used in pIε.hs3-1.2.4. These two promoters were chosen because it was thought there might be difference in activity when a promoter from the upstream cluster of constant regions was used versus one from the downstream cluster. Also, when the CL-01 cells are treated with TCDD previous results have shown a reduction in IgG secretion. When these cells are stimulated by CD40L and IL-4 a bona fide class switch to ε transcription can be observed, so it's thought that the *3'IghRR* is actively engaged with these two promoters in the CL-01 cell

line. It was hoped that some difference in activity would be observed between the three promoters reflecting differential regulation of the isotypes by the *3'IghRR*.

Results from transfection studies using the pV_H.hs3-1.2 and pV_H.hs3-1.2.4 plasmids in the CL-01 cells were very similar. The plasmids did not react much to either B-cell stimulation or TCDD treatment, whereas the hs1.2 reporter reacted strongly to both. One explanation for this could be that some portions of the *3'IghRR* might have opposing regulatory roles in human cells so adding the extra sequence could have neutralized whatever impact on activity each of the individual elements was contributing. This explanation seems insufficient, however, because when these new reporters were transfected into mouse B cells their activity was similar to the original predicted results. In mouse cells, their activity increased with stimulation and decreased with TCDD. Perhaps the mouse and human *3'IghRR*s are controlled differently. After all, the mouse sequence contains a different complement of transcription factor binding sites and is missing the hs1.2 polymorphism, so this might be a case where results from the animal model simply don't apply to the human system. If that's true, than much of what was assumed to be true about the human *3'IghRR* could be inaccurate. A complicating factor is that the 15 kb of sequence between hs1.2 and hs4 is missing in these plasmids, and if that sequence is functional the results from these studies might not reflect the activity of the endogenous locus. Unfortunately, transfection studies utilizing versions of these plasmids under the influence of the intronic promoters resulted in activity that was either very similar to the V_H plasmids or were too inconsistent to contribute to this analysis. These studies simply highlight the need to

move away from reporter plasmids and begin studying the endogenous human *3'IghRR* directly. Fortunately, recent advances in gene editing technology have finally made it possible to do so.

The development of CRISPR-mediated gene editing allows for direct study of the human *3'IghRR* for the first time. Previous genetic engineering technologies would have been too cumbersome, expensive, or inaccurate to effectively make the targeted genetic alterations necessary to study essential elements of the human *3'IghRR*. The extremely high degree of homology between the α_1 and α_2 *3'IghRR*s makes it difficult to specifically edit either one or the other, but since CRISPR only requires a 20 bp targeting sequence it should be possible to specifically edit either *3'IghRR*. There are small stretches of DNA sequence that are specific to either the α1 or α2 *3'IghRR* at both the 3' and 5' ends of each regulatory region, which means that it should be possible to selectively delete either the α_1 or α_2 *3'IghRR* by using CRISPR to induce a double strand break on either side of it. This method was employed to attempt a deletion of the α_1 *3'IghRR*.

Since the commercially available CRISPR plasmid utilized for this project contains only a single targeting sequence it was necessary to use two CRISPR plasmids to target either end of the α_1 *3'IghRR*. This was a serious problem since the CL-01 human B cell line is resistant to most transfection techniques. Typical transfection efficiencies for this cell line are less than 10% so the likelihood of co-transfecting any single cell with two plasmids is very low. Furthermore, even if a cell is successfully double transfected the success rate of creating large deletions using CRISPR can also be quite low. Never the

less, this method was attempted to delete the entire 20 kb of sequence in the α_1 *3'IghRR*. After transfecting CL-01 cells with the CRISPR plasmids clonal populations were derived by limiting dilution, DNA was extracted, and 105 clones were screened for the deletion by PCR. All 105 clones appeared to be WT cells based on the PCR results. This was not unexpected given the very low probabilities at play between the co-transfection and a potentially low rate of successful deletion by CRISPR.

A second method was employed to attempt this large deletion of the α_1 *3'IghRR*. Rather than using a standard expression vector for the CRISPR plasmid, lentiviral vectors were employed. It was hoped that the CL-01 cells could be infected with a lentivirus at a much higher rate than was possible with a standard transfection. Following infection of the CL-01 cells and a limiting dilution 189 clonal populations were screened for the deletion by PCR. Once again, all were WT cells. It's possible that the rate of successfully double infected cells, combined with the rate of successful CRISPR-mediated deletions, was still too low to capture an edited clone. Another explanation could be that the targeting sequences used in the CRISPR plasmids were not specific enough to make double stand breaks at both of the required locations. Sequencing the *3'IghRR* has always been problematic due to its complexity, repetitiveness, high G-C content, and homology between the two *3'IghRR*s. So, perhaps the targeted sequence was slightly different in the CL-01 cell line than the predicted sequence used for designing the targets. If this experiment were repeated using additional targeting sequences at both ends of the α_1 *3'IghRR*, creating this knock out cell line could still be possible.

Taking a more narrow focus, and attempting to edit the hs1.2 enhancer rather than the entire *3'IghRR*, presents a better opportunity to create genetically modified CL-01 cells. In this instance, the repetitive nature of the *3'IghRR* can be used to our advantage. The polymorphic invariant sequence within the hs1.2 enhancer is of particular interest because this polymorphism has been statistically associated with a host of immunological diseases in humans (Aupetit et al., 2000; Cianci et al., 2008; Frezza et al., 2004; Frezza et al., 2009; Frezza et al., 2007; Frezza et al., 2012; Giambra et al., 2009; Tolusso et al., 2009). Since this polymorphism is composed of 53 bp of DNA that are repeated one after another, the hs1.2 polymorphism can be targeted multiple times with a single CRISPR plasmid. However, because the α_1 and α_2 hs1.2 enhancers are nearly identical a single CRISPR plasmid will not be able to distinguish between them and both will be targeted. The α_1 hs1.2 enhancer contains two repeats of the polymorphic IS and can be cut twice by a single CRISPR plasmid, creating an enhancer with a single incidence of the IS. The α_2 hs1.2 enhancer has four repeats of the IS, but because the first one is not entirely conserved the α_2 hs1.2 can only be cut three times via CRISPR. Therefore, the α_2 hs1.2 can be reduced from four repeats of the IS to either three or two repeats. The experiment was performed using two different CRISPR plasmids (transfected into separate pools of cells), one targeting the NF1 binding site and the other targeting the DRE binding site. The plasmid targeting the NF1 binding site resulted in successfully edited cells roughly 20% of the time whereas the DRE-targeting CRISPR results in edited cells roughly 15% of the time, highlighting the need to use multiple targeting sequences when attempting to make genetic edits using CRISPR.

Analysis of the edited clones leaves no doubt that editing the hs1.2 polymorphism can have a severe impact on Ig expression. Every edited clone expressed at least one Ig isotype differently than unedited WT cells. In many cases, the edited clones expressed secreted IgM at the same level as WT cells in both stimulated and unstimulated conditions. Several clones exhibited dramatically reduced IgG secretion and appeared to be unable to increase IgG secretion with B-cell stimulation. Interestingly, the only clone that had reduced transcription of every Ig isotype is also the only clone where both the α_1 and α_2 hs1.2 enhancers had been edited. This could mean that reducing the number of IS repeats in both enhancers results in decreased Ig expression across the entire locus. That would be consistent with the theory that transcription factor binding of the hs1.2 enhancer is the driving force behind *3'IghRR* activity. Logically then, if some of these binding sites are removed there would be lower overall activity. However, when only one of the two hs1.2 enhancers is edited some of the Ig isotypes are expressed at higher levels. One possibility is that it's the ratio of certain transcription factors bound to the enhancer that is most important to hs1.2-mediated transcription. Removing one repeat from either hs1.2 enhancer but leaving the other enhancer unedited would change the relative number of transcription factors bound. Several of the transcription factors that bind to the hs1.2 enhancer are known to interact with each other, either directly or indirectly, so the alteration of the ratio of bound α_1 hs1.2 transcription factors to bound α_2 hs1.2 transcription factors could lead to specific changes in activity of the enhancers by changing the availability of transcription factors to the promoters.

Based on mouse studies, it is likely that the human *3'IghRR* engages in long range looping to interact with the V_H promoter as well as the intronic IGH promoters (Ju et al., 2007; Montefiori et al., 2016). As such, it is also likely that three dimensional conformational changes to the IGH locus are highly important to overall Ig expression. It's possible that alterations to the hs1.2 polymorphism effect the conformation of the locus through an interaction between the two *3'IghRR*s. If the hs1.2 transcription factors are interacting with each other, the two hs1.2 enhancers might interact with each other as much as with the promoters. If this is the case, it would be easy to understand how a change in the number of IS polymorphisms can have a large impact on IGH expression. Reducing the number of available transcription factor binding sites would be like reducing the amount of adhesive on a piece of tape, leading to slippage or lost binding altogether.

It was thought that certain combinations of the hs1.2 polymorphism would correspond to the expression of certain isotypes, but this data cannot support that conclusion. Expression of IGH did not seem to correlate to any particular combination of polymorphisms and two clones that had the same genotype had different IGH expression patterns. There are ambiguities in this model system that might be obscuring any direct relationship between hs1.2 polymorphism genotype and Ig expression. The CL-01 cells have a chromosomal translocation of the *3'IghRR* to the c-myc oncogene which contributes to their immortalization. So, a functional and non-functional *3'IghRR* are present in the cells and the CRISPR gene editing would target both. At this time, it is unknown if the functional or non-functional allele has been edited for any given clone.

Even if it appears that both alleles have been edited, for example if the genotyping shows four repeats for α_2 and one for α_1, there could be indel mutations in either allele not visible on the agarose gel. So that four repeats in the α_2 might appear to be a WT hs1.2 enhancer but it could actually have a disrupted binding site due to an indel mutation at the CRISPR cut site. Unfortunately, it would be impossible to know if such a mutation had occurred in the functional or non-functional allele without more thorough sequencing than is currently available for this project.

One of the more significant findings from editing the hs1.2 enhancer comes from clone 2-F3, which appears to have had the α_1 hs1.2 enhancer completely deleted. If this proves to be the case (efforts to confirm the deletion are underway) then it would reveal a surprising role for the α_1 hs1.2 enhancer in the expression of the downstream cluster of constant regions. It had been assumed that the α_1 *3'IghRR* controlled expression of the upstream constant regions and the α_2 *3'IghRR* controlled expression of the downstream constant regions. These results call that assumption into question because in the absence of the hs1.2 enhancer clone 2-F3 expressed virtually no ε transcripts. This finding could be of clinical significance because blocking the activity of the α_1 hs1.2 enhancer could reduce IgE expression and help reduce symptomology of autoimmune disorders and allergies. However, as described previously, although the α_2 hs1.2 enhancer appears to be WT (four IS repeats) it might actually have an undetectable mutation, which could account for the low ε expression. Further analysis of these genotypes must be done to provide a more thorough analysis of hs1.2 enhancer activity in these clones.

Conclusions and Future Directions

In conclusion, the 53 bp hs1.2 polymorphism has a profound influence on the expression of IGH isotypes, although the precise mechanism whereby the hs1.2 polymorphism influences Ig expression and leads to human disease states remains elusive. It is also clear that CRISPR-mediated gene editing is a viable method for studying the activity of the *3'IghRR*. The development, and further refinement, of this technology allows for the unprecedented direct analysis of the human *3'IghRR* without the need for luciferase reporter plasmids. Further work on the edited clones should investigate their sensitivity to TCDD treatment. Since specific aim 1 has revealed the NF1 binding site to significantly influence TCDD sensitivity of the hs1.2 enhancer, any clone that has an indel mutation in the NF1 binding site might have a lower sensitivity to TCDD. But since the NF1 binding site is found within the hs1.2 polymorphism any of the edited clones might exhibit altered TCDD sensitivity. Advances in sequencing technology should make it possible to further elucidate the specific genotypes of the edited clones, which might shed more light on the relationship between the hs1.2 polymorphisms and IGH expression. This project should also be repeated in other human B-cell lines since cultured, immortalized cells can sometimes behave abnormally. However, any other cell line used in this type of experiment needs to have maintained the ability to undergo class switch recombination in order to fully explore *3'IghRR* function.

VI. Materials and Methods

Model Systems and Culture Conditions

The CH12.LX cell line was generously supplied compliments of Dr. Geoffrey Haughton (University of North Carolina, Chapel Hill, NC). The CL-01 human cell line was purchased from Novus Biologicals (Littleton, CO). Cells were cultured at 37°C in a 5% CO_2 atmosphere and grown in RPMI-1640 (Mediatech, Herndon, VA) enhanced with 2 mM L-glutamine, 10% bovine calf serum (Hyclone, Logan, UT), 13.5 mM HEPES, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol and 23.8 mM sodium bicarbonate.

Specific Aim 1 Reporter Plasmid Constructs

The human polymorphic plasmids were constructed using a pGL3 basic luciferase reporter backbone (Promega, Madison, WI) containing ampicillin resistance and the luciferase gene. The enhancerless variable heavy chain promoter (V_H) plasmid and the α_{1A} plasmid were a generous gift from Dr. Michel Cogné (Université de Limoges, France) (Denizot et al., 2001; Fernando et al., 2012). Site-directed mutagenesis of the α_{1A} plasmid was performed as described previously (Fernando et al., 2012). Plasmid DNA was isolated from transformed colonies and sequenced to ensure quality and accuracy of the mutations (Retrogen, Inc., San Diego, CA). Mutations included the AP-1, NFκB, DRE, Oct, AP1.ETS, NF1, 5' SP-1 or 3' SP-1 sites and were termed α_{1A}AP1mut, α_{1A}NFκBmut, α_{1A}DREmut, α_{1A}Octmut, α_{1A}AP1.ETSmut, α_{1A}SP-1.1mut and α_{1A}SP-1.2mut, respectively (Table 1). Additionally, the invariant sequence (IS) was deleted from the α_{1A}

(α_{1A}ISdel) and mutants containing the IS deletion plus the Oct mutation (α_{1A}ISdelOctmut) as well as a DRE and NF-κB mutation (α_{1A}DRE.NFκBmut) were also generated. Mutations of transcription factor binding sites were designed based on previous electrophoretic mobility shift assay data and motif analysis with TFSEARCH, an online transcription factor profile database (Grant et al., 1995; Heinemeyer et al., 1998; Lenardo, Pierce, & Baltimore, 1987; Yao & Denison, 1992).

Specific Aim 2 Reporter Plasmid Constructs

The entire 5 kb sequence from hs3 to hs1.2 was constructed by a commercial gene manufacturing company called Genscript (Piscataway, NJ). The sequence used for the plasmid was based on human sequencing results generously provided by Dr. D. Frezza (University of Tor Vergata). This sequence was inserted into the enhancer region of a pGL3 luciferase reporter containing the V_H promoter and could be used in experiments without the hs4 enhancer if desired. In step-wise fashion, the hs4 enhancer was then inserted 3' of the hs1.2 enhancer, but without any intervening sequence. Restriction digests, as well as sequencing results, confirm the presence of the inserted *3'IghRR*. Two promoters were selected for insertion into the plasmid. One was the γ_3 promoter, which contains a potential DRE, AhR binding site. The other was the ε promoter. The sequences for these promoters were constructed by Genscript, based on published papers, and inserted into the multiple cloning site (Hu et al., 2000; Kim, Edmonston, Wu, Schaffer, & Casali, 2004).

Specific Aim 3 CRISPR Plasmids

CRISPR plasmids were purchased from Origene (Rockville, MD) in either a standard plasmid form (pCas-Guide-EF1a-GFP) or as a lentiviral vector (pLenti-Cas-Guide). The plasmids were cut using restriction enzymes BamHI and Esp3I (an isoschizomer of BsmBI) and the cut plasmids were purified from a 1% agarose gel in preparation for cloning the targeting sequences (Table 2). The targeting sequences were inserted into the cut plasmids using T4 ligase (Promega) and the ligated plasmids were transformed into chemically competent *E. coli* (Zymo). Isolated colonies were plucked from an LB agar plate, grown in a 3 ml culture using LB broth, and the plasmids extracted via miniprep (Zymo). The minipreps were screened for successful insertion of the targeting sequence by DNA sequencing (Retrogen). When a successfully cloned plasmid was identified a transfection-quality preparation of the plasmid was created using an endotoxin-free Qiagen maxiprep kit.

Use of the CRISPR Plasmids

The pCas-Guide-EF1a-GFP plasmid was transfected into CL-01 cells as previously described. The transfected cells were allowed to incubate for 72 hours at a concentration of 1 x 10^5 cells/ml and were then sorted for GFP expression by the Research Flow Cytometry Core at Cincinnati Children's Hospital. Cells expressing GFP were seeded into a 96 well plate at 1 cell/well. When the cells outgrew the 96 well plate they were transferred to a 24 well plate. When sufficient cells numbers were achieved

to sample their DNA, a portion of the cells were used in a genomic DNA extraction (Zymo) which was then used in a genotyping PCR.

Following insertion of the targeting sequence, the pLenti-Cas-Guide plasmid was packaged into infectious lentiviral particles by the Cincinnati Children's Hospital viral core. Two separate viruses were produced; one which targeted the 5' end of the α_1 *3'IghRR* and one which targeted the 3' end. Equal volumes of the two viruses were mixed together and 500 µl of this mixture was used to re-suspend 2×10^6 CL-01 cells, which were incubated for 6 hours in the viral mix. After the incubation period, another 500 µl of the viral mix was added to the cells and they were incubated overnight. The next day, the infected cells were seeded in a 96-well plate at a concentration of 0.5 cells/well. Eight 96-well plates were prepared in total. Half of the plates contained 4 µg/ml polybrene. Two plates which contained polybrene and two that did not were treated with additional 10 µl of viral mix. All the plates were returned to the incubator and the cells were permitted to grow until DNA could be isolated for genotyping as described previously.

Genotyping the Genetically Edited Cells

To genotype the hs1.2 enhancer 100 ng of DNA was added to a PCR mix containing 1x standard taq buffer (NEB), 10 mM dNTPs (ThermoFisher), 10 µM forward and reverse primers (Table 1, Eurofins), standard taq (NEB), and nuclease-free water. Cycling conditions followed a touchdown PCR format with an initial denaturation of 95°C for 30 seconds followed by a three step cycle of 95°C for 30 seconds, 75°C for 45

seconds, and 68°C for 30 seconds repeated 8 times with the annealing temperature decreasing 1°C after every cycle. After 8 cycles, the program changed to a new three step cycle of 95°C for 30 seconds, 67°C for 45 seconds, and 68°C for 30 seconds repeated for an additional 27 cycles followed by a final extension step of 68°C for 5 minutes. Expected product sizes are 209 bp for one IS repeat, 264 bp for two repeats, 319 bp for three repeats, and 374 bp for four repeats. Products were separated on a 2% agarose gel.

To genotype for the presence/absence of the α_1 *3'IghRR* following attempts to delete it 100 ng of DNA was added to a PCR mix containing 1x standard taq buffer (NEB), 10 mM dNTPs (ThermoFisher), 10 µM forward and reverse primers (Table 1, Eurofins), standard taq (NEB), 3% DMSO, and nuclease-free water. Cycling conditions were 95°C for 30 seconds followed by a three step cycle of 95°C for 30 seconds, 62°C for 30 seconds, and 68°C for 30 seconds repeated 40 times followed by a final extension step of 68°C for 5 minutes. Two products are expected for wild type cells of 500 bp for a product encompassing the 5' CRISPR targeting site and 400 bp for the 3' targeting site. If the α_1 *3'IghRR* is successfully deleted a single product of 300 bp will form. PCR products were separated on a 2% agarose gel.

Sequencing the Genotyping PCR Products

Sequencing of all genotyping PCR products was attempted, but success was limited. Three methods were employed to sequence the products. In the first method, the PCR products were purified from the agarose gel and then cloned into the pGEM-T

Easy vector (Promega). The cloned vector was transformed into bacteria and isolated colonies representing a single PCR product were cultured, miniprepped, and the resulting plasmid DNA sequenced by Retrogen, Inc. The second method employed was the same except the products were not separated on a gel. Instead, an enzymatic clean-up called ExoSAP-IT (Affymetrix) was performed to digest all unincorporated nucleotides and the resulting mixture was cloned into the pGEM vector. The final method involved extracting the separated products from a gel and attempting to sequence the product directly without cloning it into a vector.

Stimulation of hs1.2 Edited Cells

CL-01 cells which were successfully edited in the hs1.2 enhancer were assessed for antibody expression following stimulation by CD40 ligand and IL-4. The CD40 ligand was used at a concentration of 6.25 ng/ml and the IL-4 was used at 25 ng/ml. Cells were treated at a concentration of 1×10^5 cells/ml and incubated for 96 hours. After incubation, the cells were pelleted by centrifugation and the supernatant was used for an ELISA while RNA was extracted from the cell pellet.

RNA Extraction and Real Time PCR

RNA was extracted from the cells using Tri-Reagent (Sigma) according the manufacturer's protocol. The RNA was converted to cDNA using a cDNA synthesis kit (Bio-Rad). Real time PCR was performed on 100 ng of cDNA using the Luminaris Real

Time Mastermix (Thermo) and cycling conditions followed the manufacturer's recommendations. Primers used for RT-PCR can be found in Table 1.

Sandwich ELISA

ELISAs were performed using supernatant from stimulated and naïve cells as described previously (Sulentic et al., 1998, 2000). Briefly, 100 µl of supernatant was added to a 96 well plate coated with a human Ig capture antibody. A secondary antibody specific to either IgM or IgG and bearing an HRP tag was then added to the plate after incubation and washing. Colorimetric analysis followed the addition of TMB and sulfuric acid.

Transient Transfections and Luciferase Assays for Reporter Plasmids

Transient transfections and luciferase assays were performed as described previously (Fernando et al., 2012; Sulentic et al., 2004). Briefly, 1 x 10^7 CH12.LX or CL-01 cells were transfected with 10 µg of reporter plasmid by electroporation then aliquoted into appropriate treatment groups and incubated for 24 hours at a seeding concentration of 2 x 10^5 cells/ml. Following incubation, the cells were separated from the media by centrifugation and resuspended in reporter lysis buffer (Promega, Madison, WI) and frozen for at least one hour at -80^{o}C. After thawing and centrifugation to remove cell debris 20 µl of sample lysate was mixed with 100 µl of luciferase substrate reagent and measured by a single-tube luminometer (Promega, Madison, WI, Berthold Detection Systems, Oak Ridge, TN). Transfection efficiency was determined

using RT-PCR on DNA extracted from transfected cells using methods described previously (Sulentic et al., 2004). The forward and reverse primers are 5'-ACTGGGACGAAGACGAACACTT-3' and 5'-TCAGAGACTTCAGGCGGTCAA-3', respectively. Sample PCR data was compared to a standard luciferase reporter plasmid with concentrations ranging from 0.1 ng/µl to 1x10-6 ng/µl. Amount of transfected plasmid (ng) was calculated by taking the concentration of DNA from the PCR results (ng/µl) x the volume of DNA added (2µl) x the fold dilution (Fernando et al., 2012). Number of plasmids per cell was calculated from the equation: [ng of plasmid DNA x number of plasmids/ng] ÷ total number of cells isolated (previously described in (Sulentic et al., 2004)). Luciferase activity was normalized to plasmids per cell.

Statistical Analyses of Data

Comparisons between treatment groups (n=3) of the same reporter plasmid were analyzed using a 1-way ANOVA followed by a Dunnett's Multiple Comparison post test. Significant differences in luciferase activity (mean ± SEM) normalized to transfection efficiency were compared to the corresponding vehicle control and represented by "*", "**", "***" at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Differences in TCDD-induced fold change compared to vehicle control was determined in the same manner and denoted by "‡", "‡‡", "‡‡‡" at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Differences in luciferase activity or fold change between reporter plasmids were analyzed using a 2-way ANOVA with Bonferroni post test. Daggers, "†", "††", "†††", denote significant differences between different reporter plasmids at $p<0.05$, $p<0.01$

and $p<0.001$, respectively. The TCDD-induced fold change results represent the mean fold change from at least three separate experiments (mean ± SEM). RLU data was normalized to transfection efficiency.

Mutation	Forward Primer Sequence
α_{1A}+Pax5	AGGCCCACTTGGGTTA**GGGCAAGGATGGGTGGCTGCACACCCCAGGG**CTGACACTGGGACCAC
α_{1A}AP1mut	CTCCCCCAGCGTGGCCAGGC**ATCTAAG**GGCCTCCAGATTCGGGGACA
α_{1A}NFκBmut	CTGGCTCAGGCCTCCAGATT**GTAGAAGAACT**GCCCCCTCCCCCAGGACAGG
α_{1A}DREmut	CCCGCCCCCTCCCCCA**AGTCT**GCCAGGCTGGCTCAGG
α_{1A}Octmut	TCTGGGCAGACTTGGCCCTCAGGGCAC**CCTAAGTC**GGTTGTTTGTTCCACACCGAAAACATG
α_{1A}ISdel	CACGATTCTCCCGCCCCCTCCCCC
α_{1A}AP1.ETSmut	GGCTCTGATCTGTTTCTCCTTGAG**GGGCCAACTAG**GGGCAGACTTGGCCCTCAGGGCAC
α_{1A}SP1.1mut	CCAGAAATAGCTTGCACGATTCT**AGTCGAT**CCTCCCCCAGCGTGGCCAGGCTG
α_{1A}SP1.2mut	GCCTCCAGATTCGGGGACA**AGTCGAT**CCTCCCCCAGGACAGGCCC
α_{1A}NF1mut	AGCGTGGCCAGGCTGGCTC**TTAAGTTG**GATTCGGGACACCCGCCC
α_{1A}DRE.NFkBmut	Serial mutagenesis performed using primer sets from α_{1A}DREmut and α_{1A}NFκBmut
α_{1A}ISdelOctmut	Serial mutagenesis performed using primer sets from α_{1A}ISdel and α_{1A}Octmut

Table 1. Primers used for site-directed mutagenesis of the hs1.2 reporter plasmid. Sequences listed in the table are forward primers. Reverse primers are the reverse complement of the forward primer. Nucleotides that were mutated are bold and underlined.

CRISPR Target	Targeting Sequence
Alpha1-5'Trgt	GTCCTAACTCAGTCCTGGTC
Alpha1-3'Trgt	CCGATCCTGTCGCCATAGTG
NF1cut	CAGCGTGGCCAGGCTGGCTC
DREcut	GACACCCCCCCACCACAGCG

Table 2. CRISPR/Cas9 targeting sequences. These targeting sequences were cloned into the CRISPR plasmids (Fig. 13) following the manufacturer's instructions. "Alpha1-5'Trgt" and "Alpha1-3'Trgt" represent the sequences which bind to the 5' and 3' end of the α_1 *3'IGHRR*, respectively. "NF1cut" targets the polymorphism within the hs1.2 enhancer and introduces a double strand break in the NF1 binding site. "DREcut" is the same, except it creates a break in the DRE binding site. The NF1 and DRE targeting CRISPRs were used independently and generated similar results, although the DRE CRISPR created edited clones with less efficiency.

VII. References

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